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REMARKS

Support for this amendment is found throughout the specification, for example, at page 11, line 30 through page 13, line 18. No new matter enters by this amendment. Accordingly, Applicants respectfully request entry of this response, reconsideration of the application, and timely issuance of a Notice of Allowance.

Applicants acknowledge with appreciation the withdrawal of the rejection of claims 15-16 and 18-20 under 35 U.S.C. § 103 over Barre-Sinoussi et al.; the rejection of claims 15-16 and 8-20 under 35 U.S.C. § 101; the rejection of claims 16, 19, 30, and 31 under 35 U.S.C. § 102(a) over McDougal et al.; the rejection of claims 15-16, 18-20, and 29-31 under 35 U.S.C. § 103 over Luciw and Dina; and the rejection of claims 15-16, 18-20, and 29-31 under 35 U.S.C. § 103 over McDougal et al. and Barre-Sinoussi et al.

The specification is objected to and claims 29-31 remain rejected under 35 U.S.C. § 112, first paragraph, as the specification allegedly does not provide support for the invention as claimed. In particular, the Examiner asserts that the term "immunological complex" as recited in claims 29-31 is not supported. Applicants respectfully traverse the rejection.

The rejection concedes that "immunocomplexes" and "immune complexes" are explicitly cited in the specification. (Paper No.27 at 1.) However, the Examiner states that

> . . . this brief recitation is not sufficient to provide support for the instantly claimed immunological complexes involving the viral antigens p12 or p18 and antibodies directed against said antigens. The specification does not disclose the contemplation, preparation, purification, or associated use of these immune complexes.

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(Paper No. 27 at 1-2.)

Under the written description requirement of 35 U.S.C. § 112, first paragraph, applicants must convey with reasonable clarity to those skilled in the art that, as of the filing date sought, they were in possession of the invention, i.e., whatever is now claimed. *Vas-Cath Inc. v. Mahurkar*, 19 U.S.P.Q.2d 1111, 1117 (Fed. Cir. 1991). The standard for determining sufficiency of the description is "factual and depends on the nature of the invention and the amount of knowledge imparted to those skilled in the art by the disclosure." In re Wertheim, 541 F.2d 262 (citing In re Ruschig 379 F.2d 990, 995-96 (C.C.P.A. 1967)).

The specification provides ample support and guidance for the preparation, purification, and use of the claimed immune complexes, especially in view of the "knowledge imparted to those skilled in the art." For example, the specification teaches how to purify the proteins, p18 and p12, and how to elicit an antigen-antibody response in sera of patients suffering from or having LAS or AIDS. (Specification, page 13, third paragraph.) Applicants further describe the immunoprecipitation and analysis of immune complexes in accordance with the invention. (Specification, page 12, first full paragraph.) At page 17, line 19, through page 18, line 10, applicants describe assays where one skilled in the art would understand that the immunological complexes were present. Moreover, at page 37, the legend of Figure 5 describes the method of isolating immune complexes by protein A Sepharose beads. Original claim 1 further particularly points out the known utility of the immune complexes for diagnostic purposes. Therefore, one

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having skill in the art would have been guided by the specification to make and use the claimed invention.

Moreover, contemplation of immune complexes as part of the invention is further evidenced by the skill in the art at the time the application was filed. For example, Jones and Orlans, *Journal of Immunological Methods*, 44:249-270 (1981)(Exhibit 1), reviews the characterization of immune complexes and their uses around that time. Therein, the authors teach that:

> Soluble immune complexes (IC) circulating in the blood stream can be detected by a variety of methods and these are widely used in clinical immunology to aid the study of immunopathology of diseases, the assessment of disease progression and the patient's response to therapy. . . If the antigen or the specificity of the antibody could be identified some indication of the aetiology of the disease and a pathological role for the complexes might be obtained. Moreover there are instances where IC are found in sera but neither free antigen nor antibody can be detected and in these patients the isolation and identification of the IV might be of diagnostic importance.

(Jones and Orlans, page 249, first paragraph.) The reference further describes methods of handling, preparing, isolating, and using immune complexes.

In addition, Higgins, Experentia 36:889-890 (1980)(Exhibit 2), further exemplifies the skill in the art with regard to immune complexes. Higgins describes the immunogenicity of agarose-immobilized immune complexes in rabbits. Thus, the use of an immune complex as an antigenic determinant was known in the art.

In addition, Harlow et al., Antibodies: A Laboratory Manual (1988)(Exhibit 3), teach at page 464 that "[f]orming an antibody-antigen complex is the simplest step in an

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immunoprecipitation." The authors go on to provide well-known methods of making and purifying immune complexes. See Harlow et al., pages 464-468. Moreover, Harlow et al. teach the use of immune complexes as antigens: "Antigens purified by immunoprecipitation often show enhanced immunogenicity. . . Purified immune complexes can be injected directly or can be injected coupled to beads." See Harlow et al., page 135. The authors further describe the steps for preparing immune complexes for injection as antigens. See Harlow et al., pages 136-137.

As is clearly established by Jones and Orlans, Higgins, and Harlow et al., one having ordinary skill in the art would immediately appreciate how to make, purify, and use the claimed immune complexes. An applicant preferably omits descriptions of well-known techniques from a patent specification. Spectra-Physics, Inc. v. Coherent, Inc., 827 F.2d 1524, 1534, 3 U.S.P.Q.2d 1737, 1743 (Fed. Cir. 1987), *citing* Hybritech Inc. v. Monoclonal Antibodies, Inc., 802 F.2d 1367, 1384, 231 U.S.P.Q. 81, 94 (Fed. Cir. 1986) *cert. denied*, 107 S. Ct. 1606 (1987). The present specification conforms to that preference.

Thus, in view of the knowledge of one skilled in the art and applicants' disclosure, the specification clearly conveys the invention of claims 29-31 to one having skill in the art that the inventor had possession of the claimed invention at the time of the filing date. In particular, it is clear that the inventors clearly contemplated the immune complexes associated with p12 and p18 of HIV-1 and that one having skill in the art would have been capable of making, purifying, and

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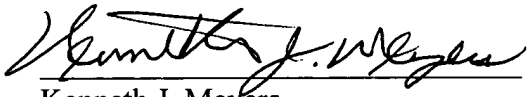
using the immune complexes. Accordingly, applicants respectfully request reconsideration and withdrawal of the rejection.

In the event that the Examiner disagrees, he is invited to call the undersigned to discuss the remaining issue in this case to expedite prosecution.

If there are any other fees due in connection with the filing of this Amendment, please charge such fees to our Deposit Account No. 06-0916. If an extension of time is required under 37 C.F.R. § 1.36 and not accounted for above, such an extension is respectfully requested and the fee should be charged to Deposit Account No. 06-0916.

Respectfully Submitted,

FINNEGAN, HENDERSON, FARABOW,
GARRETT & DUNNER, L.L.P.



Kenneth J. Meyers
Registration No. 25,146

Date: October 28, 1996

Encls: 1) Exhibit 1: Jones and Orlans (1981)
2) Exhibit 2: Higgins (1980)
3) Exhibit 3: Harlow et al. (1988)

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WASHINGTON, DC 20005
202-408-4000

AL METHODS

30 July 1981

...g monoclonal antibodies to cell ... 125

...s ... 135

...using complement-mediated im... 153

...ytes isolated by centrifugation in ... 159
...myocarditis
...e, D. Raiklen, D.A. Carson, J.H. ...

...immunoglobulin coated Petri dishes 171

... 183

...ng of eosinophils by fluorescein... 191

...a proteins 199

...liferation of rat lymph node cells 205
...t, R.C. Aalberse
...ating immune complexes 211
...in
...glycosphingolipids with blood group ...
...cromethod based on inhibition of ... 223

...ed hemadsorption 235

...the central artery of the ear 241

247

248

...tracts, Index Medicus, Chemical Abstracts, ...
... Contents/Life Sciences, Excerpta Medica, Informedicus)

immunological methods

LAST ISSUE OF THIS VOLUME

Editors:

E.J. HOLBOROW
V. NUSSENZWEIG

Volume 44, No. 3, 1981

...69
...) 249–372 (1981)

ELSEVIER/NORTH-HOLLAND

JOURNAL OF IMMUNOLOGICAL METHODS

Vol. 44 No. 3 14 August 1981

CONTENTS

Review article

V.E. Jones, E. Orlans
Isolation of immune complexes and characterisation of their constituent antigens and antibodies in some human diseases: a review 249

Research reports

R.C. Birkmeyer, L.L. Keyes, A.L. Tan-Wilson
Determination of relative antigen-antibody affinities by quantitative immunoelectrophoresis 271

P.C. Dubreuil, C. Foa, J.-R. Galindo, D.H. Caillol, F.A. Lemonnier
Cell surface fixation of alloantigen bearing plasma vesicles in the presence of polyethylene glycol 285

D.G. Kilburn, G. Shematek, S. Crane
Quantitation of helper factor activity. I. Utilization of the ELISA method to measure helper activity in the secondary antibody response 301

A. Aitkaci, J.C. Monier, N. Mamelle
Enzyme-linked immunosorbent assay for anti-histone antibodies and their presence in systemic lupus erythematosus sera 311

P. Borzini, F. Tedesco, N. Greppi, P. Rebulla, A. Parravicini, G. Sirchia
An immunoenzymatic assay for the detection and quantitation of platelet antibodies: the platelet β-galactosidase test (PGT) 323

H.D. Todd-Kulikowski, R.G. Parsons
A stable, sensitive assay for human fibronectin 333

H. Elwing, M. Stenberg
Biospecific bimolecular binding reactions – a new ellipsometric method for their detection, quantification and characterization 343

A.C.J.M. Antonissen, K.P.M. Van Kessel, H. Van Dijk, J.M.N. Willers
Development of a simple passive haemagglutination-inhibition assay for *Listeria monocytogenes* lipotheichoic acid 351

Book review 359

Contents to Volume 44 (1981) 361

Author Index to Volume 44 (1981) 365

Subject Index to Volume 44 (1981) 369

(Cited in: *Biological Abstracts, Index Medicus, Chemical Abstracts, Current Contents/Life Sciences, Informedicus, Excerpta Medica*)

Journal of Immunological Methods, 44 (1981) 249–270
Elsevier/North-Holland Biomedical Press

Review article

ISOLATION OF IMMUNE COMPLEXES AND CHARACTERISATION OF THEIR CONSTITUENT ANTIGENS AND ANTIBODIES IN SOME HUMAN DISEASES: A REVIEW

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(Accepted 9 April 1981)

(1) INTRODUCTION

Soluble immune complexes (IC) circulating in the blood stream can be detected by a variety of methods and these are widely used in clinical immunology to aid the study of immunopathology of diseases, the assessment of disease progression and the patient's response to therapy (e.g., Pussell et al., 1978; Hay et al., 1979; extensively reviewed by Theofilopoulos and Dixon, 1979). Most of these methods show the presence of IC, some provide semi-quantitative estimates but they do not identify the constituent antigen and antibody in the complexes (Lambert et al., 1978). If the antigen or the specificity of the antibody could be identified some indication of the aetiology of the disease and a pathological role for the complexes might be obtained. Moreover there are instances where IC are found in sera but neither free antigen nor antibody can be detected and in these patients the isolation and identification of the IC might be of diagnostic importance (Levo et al., 1977; Kazatchkine et al., 1980; Roberts and Lewis, 1980).

In this review we discuss methods for the isolation and purification of immune complexes from biological fluids in amounts sufficient to analyse.

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Abbreviations: Ab, antibody, Ag, antigen; Agg., aggregated; BSA, bovine serum albumin; C, complement; C3bi, inactivated C3b; DNA, deoxyribonucleic acid; EAV, equine arteritis virus; EDTA, ethylenediamine tetraacetate; HBsAg, hepatitis B surface antigen; HSA, human serum albumin; IC, immune complexes; Ig, immunoglobulin; μ, ionic strength; m.wt., molecular weight; NaSCN, sodium thiocyanate; NeF, nephritic factor; PBS, phosphate buffered saline; PEG, polyethylene glycol; RA, rheumatoid arthritis; RF, rheumatoid factor; RNase, ribonuclease; RNP, ribonucleoprotein; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SLE, systemic lupus erythematosus; VBS, veronal-buffered saline.

0022-1759/81/0000—0000/$02.50

We examine existing methods and new possibilities for the identification of constituent antigens and antibodies and of the other serum components almost invariably found in IC such as complement and rheumatoid factors (Casali and Lambert, 1979; Porter, 1980; Vejtorp, 1980). Although the literature on this topic is recent and limited, this survey is not comprehensive. It excludes, for example, all references to isolation of complexes from tumour patients but includes those techniques used most successfully on other clinical material.

(2) SAMPLE HANDLING

Precautions in the preparation of samples for detection and isolation of IC in serum are outlined in a WHO Technical Report (1977). Aggregated IgG behaves like IC in most of the tests used for the detection of IC and Pereira et al. (1980) thought that most aggregation of IgG occurred during blood clotting and serum preparation; blood samples therefore should clot at 37°C for 3 h, which also avoids loss of cryoglobulins. Alternatively plasma can be used but any fibrin must be removed. If samples are to be frozen, storage at −70°C is recommended because aggregation of IgG may occur during prolonged storage at −20°C. In our experience, however, insufficient aggregation occurred during 2 years at −20°C to be detected by the sensitive platelet aggregation test (unpublished). Repeated freezing and thawing should be avoided because this also causes aggregation of IgG (Capra and Kunkel, 1970).

(3) CONTAMINANTS IN PREPARATIONS OF IMMUNE COMPLEXES

Serum proteins other than the constituent antigen and antibody which may be bound to immune complexes or contaminate IC preparations will vary with the method of isolation (see Table 1). Preparations isolated by polyethylene glycol precipitation, by sucrose density gradient centrifugation and by gel filtration will be contaminated mainly with large molecular weight components (e.g., Zubler et al., 1977; Male and Roitt, 1979; Benveniste and Bruneau, 1979). Contaminants which appear in IC preparations as a direct consequence of the particular technique used to isolate the complexes will be discussed in the relevant section.

All the techniques discussed in this review isolate aggregated IgG if it is present in the initial sample. Three methods which are currently used simply to detect IC but which could be modified to isolate them, are the exceptions. It is claimed that anti-C3 will not bind aggregated IgG if EDTA is added to the blood sample immediately on collection before aggregation occurs and before the aggregates could activate C3 (Pereira et al., 1980). The second method depends on solid phase antibody specific for the antigen in the complex (Pernice and Sedlacek, 1979) and this antibody should not bind aggregated IgG or the constituents of an unrelated immune complex. The

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TABLE 1
Contaminants and constituents other than the relevant antigen and antibody which may be found in preparations of immune complexes.

Methods of isolation	Common contaminants	Contaminants peculiar to the method of isolation	References [a]
> 3% PEG precipitation	aggregated IgG	other > 7S serum proteins + 7S IgG	Zubler et al. (1977)
SDG centrifugation	complement components: C1q, C1r, C1s, C2, C4, C3	other > 7S serum proteins + 7S IgG	Benveniste and Bruneau (1979)
Gel filtration	rheumatoid factors	other > 7S serum proteins + 7S IgG	
Binding to:			
Protein A Sepharose	anti-idiotype antibodies	7S IgG1, 2 and 4; traces of IgA, IgM, IgE	Goding (1978); Inganas et al. (1980)
Protein A bacteria	unrelated immune complexes	other serum proteins bind to Sepharose	Vidal and Conde (1980)
Raji cells	C-reactive protein	antibodies to Raji cell membrane Ags; e.g. anti-HLA and anti-lymphocyte antibodies, antibodies to nuclear antigens	Horsfall et al. (1981)
C1q	other serum constituents bound covalently but not specifically to the antibody isotype	polyanions, endotoxins, some viruses, DNA	Agnello et al. (1971); Woodroffe et al. (1977); Cooper et al. (1976)
Conglutinin	immunoconglutinin	IgG antibody associated with C3 fragments	Pereira et al. (1980)
Anti-immunoglobulins		free immunoglobulin of the relevant isotype	
Rheumatoid factors		IgM, i.e. eluted IgM RF, and IgG	Gilead and Sulitzeanu (1979)
Anti-C3 or anti-C4	as above, but no agg. IgG if EDTA added during blood collection	IgG antibody associated with C3 fragments	Pereira et al. (1980)
Antibody to a specific antigen	as above; but no agg. IgG or unrelated immune complexes	free antigen	Pernice et al. (1979a)

[a] References given are examples of the most recent ones.

third method is the anti-antibody technique of Kano et al. (1978).

Certain serum proteins interact, and those such as IgA bind to other serum constituents (Heremans, 1974). For example, alpha$_1$ anti-trypsin can bind to IgA (Haines, pers. comm.) and if this IgA has antibody activity, then alpha$_1$ anti-trypsin could be covalently bound to an IgA-containing immune complex. Other IC unrelated to the disease process may be present in the patient's serum and be isolated simultaneously with the IC under study: IC have been found in infants fed on cow's milk (Delire et al., 1978) and circulating idiotype anti-idiotype complexes have been detected in normal human sera (Morgan et al., 1979). Indeed there is evidence that anti-idiotypic autoantibody is formed during any normal immune response, e.g., Goidl et al. (1980).

Autoantibodies found in IC include rheumatoid factors, immunoconglutinin and nephritic factor (NeF). Immunoconglutinin and NeF bind to complement components fixed in IC. RFs can contaminate isolated IC preparations in several ways. First, they may bind to the Fcγ region of the antibody used to immunoprecipitate or bind IC; as RFs do not bind to F(ab')$_2$ fragments of IgG this portion of a specific antibody can be used to isolate IC from clinical material which contains both RFs and IC (Pereira et al., 1980). Secondly, RFs may be bound to IgG antibody in the IC to be tested; IgM RF binds to IgG anti-nuclear antibodies in some sera from patients with connective tissue diseases (Mach et al., 1980), IgG RF is similarly bound in spontaneously autoimmune mice (Izui and Eisenberg, 1980) and IgM RF also binds to IgG anti-viral antibodies (Vejtorp, 1980). Absorption of test sera with aggregated human IgG coated on to latex paricles will remove unwanted free RFs but not necessarily those already bound to IC. Thirdly, RFs may cross-react with other antigens such as nuclear antigens (Hannestad, 1978; Johnson, 1979; Agnello et al., 1980) and could be incorporated into immune complexes of DNA-histone and anti-DNA. Immunoconglutinin, an IgM antibody against activated complement components C3b and C4, is formed in man in response to certain infections and in some patients with rheumatic diseases (Nydegger et al., 1980). NeF binds to the alternative pathway convertase C3b,Bb in IC and is found in some forms of membrane proliferative glomerular nephritis and in partial lipo-dystrophy (Sissons et al., 1976). Both immunoconglutinin and NeF would be trace contaminants in IC but RFs could be a major proportion of the immune complex protein.

Complement components which interact and bind to immune complexes (Porter, 1980) are the early component of the classical pathway, C1q, C1r and C1s and activated C4 and C2, which fuse together to form $\overline{C42}$, the classical C3 convertase, which in turn leads to the attachment of C3b to the complex. The amounts of these components in an IC preparation relative to the amount of antigen and antibody is not known precisely but could be more than 50% of the total protein. The C1 macromolecule is thought to consist of one molecule of C1q (400 000 m.wt.) and a tetramer composed

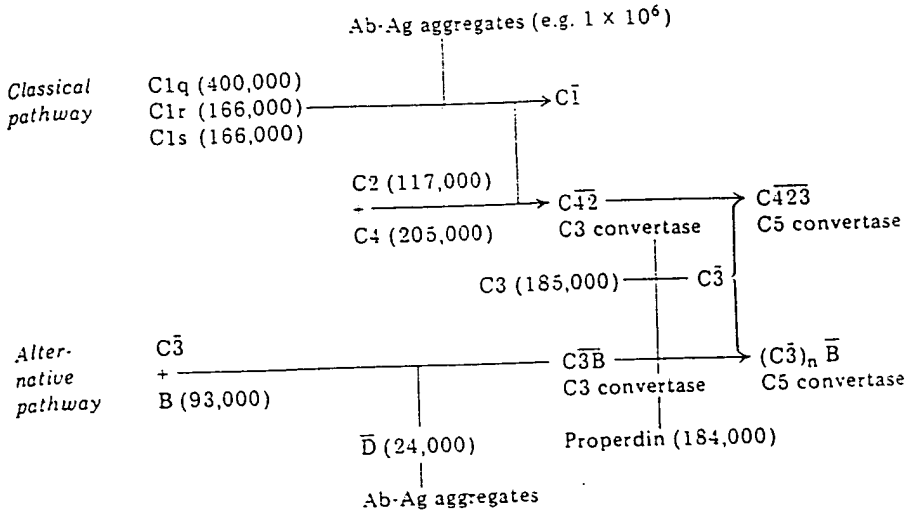

Fig. 1. A scheme of the assembly and activation of the early components of complement after their interaction with antibody-antigen complexes (molecular weights in daltons) (adapted from Porter, 1980).

of C1r dimer and C1s dimer (i.e., 4 × 83 000 m.wt.). In addition, C4, C2 and C3 have a combined molecular weight of about 0.5×10^6. Therefore the total molecular weight of these components when attached to a complex could exceed 1×10^6. A soluble immune complex will probably be composed of at least Ag_2Ab_3 before C1 can interact, be assembled and activated by the complex (Mannik et al., 1971; Mannik, 1980) but assuming a molecular weight of 100 000 for the antigen and 150 000 for IgG antibody, the AgAb complex could still be less than 1×10^6 (Fig. 1). Indeed a protein stain of SDS-PAGE to illustrate the binding of C4b to AgAb aggregates showed large amounts of stained C components compared with the amount of antibody γ and light chains (Porter, 1980).

In the alternative pathway, AgAb aggregates containing IgG4, IgA or IgE isotypes, bind the protease D̄ (24 000 m.wt.), C3̄ (≃180 000 m.wt.) and B̄ (93 000 m.wt.) stabilised by properdin (184 000 m.wt.) and therefore a considerable portion of the protein in complexes of these isotypes could be alternative pathway components (Nussenzweig, 1980). Takahashi et al. (1977) found that the molar ratio of C3b to antibody is close to 1 in some soluble complexes.

(4) CONCENTRATION OF IMMUNE COMPLEXES

Because IC are by definition larger than their constituent antigen and antibody and also are usually larger than most serum proteins, their initial separation can be achieved by density gradient ultracentrifugation, gel filtra-

tion (Amlot et al., 1976; Garvey et al., 1977; Benveniste and Bruneau, 1979) or precipitation with polyethylene glycol of molecular weight 6000. Sucrose density gradient ultracentrifugation and gel filtration are reliable methods for isolating IC in the heaviest fractions but are not widely used in clinical laboratories because the processing of large numbers of serum samples is laborious, although recently Kilpatrick et al. (1980) have separated IC from 40 sera by gel filtration on Sephacryl S300. PEG has been more widely used because large numbers of samples can be easily processed and because it was one of the earlier techniques devised for the detection of soluble IC (Harrington et al., 1971; Creighton et al., 1973). At a final concentration of 3—4% in serum diluted 1 : 3, PEG was used to precipitate soluble complexes from the sera of patients with diseases such as SLE, RA, leprosy and leukaemia (Zubler et al., 1977).

Whilst PEG is useful to concentrate IC, it is important to remember that the precipitated material can contain large amounts of other serum proteins (Zubler et al., 1977) and that the recovery of IC is only partial: for example 3.5% PEG yielded only 34% of a preparation of preformed radiolabelled complexes compared with a yield of more than 90% after gel filtration (Heimer et al., 1979). However, material concentrated by PEG may be redissolved and further purified by adsorption to conglutinin or C1q (Casali and Lambert, 1979) or by sucrose density gradient centrifugation which simultaneously removes residual PEG. Recently, in order to avoid sequential purification in an attempt to reduce unwanted dissociation of low affinity immune complexes, Male and Roitt (1979) used a single step by combining PEG and sucrose in a density gradient before centrifugation. Nonetheless the precipitated material was still heavily contaminated with other serum proteins although yields of material sedimenting at >19S were somewhat greater than after simple precipitation with 4% PEG.

(5) ISOLATION OF IMMUNE COMPLEXES

Methods for the isolation of IC are generally derived from those used for their detection but, of course, do not include those techniques which depend on inhibition of agglutination or lysis by the complexes.

(a) Staphylococcal protein A

Use of protein A as an immunological reagent was reviewed by Goding (1978). The affinity of protein A for human IgG is restricted to IgG1, 2 and 4 and the binding site is in the Fc portion of the IgG. Thus its usefulness does not extend to the preparation of complexes formed by IgG3, IgA, IgM or IgE. According to Tucker et al. (1978) small complexes formed in antigen excess which persist in the circulation should be bound as effectively as larger complexes because binding depends only on the affinity of protein A for the antibody Fc region. Initially intact staphylococci were used as a solid phase immunoadsorbent for IC (Kessler, 1975) but purified protein A

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coupled to Sepharose is now more widely used. Both are available commercially (Pharmacia, Uppsala, Sweden, and Calbiochem Behring Co., San Diego, CA, U.S.A. for protein A Sepharose and *Staphylococcus aureus* Cowan 1 respectively). Protein A Sepharose has a larger binding capacity (1 g dry weight of gel binds 80 mg IgG whereas 1 g bacteria binds only 15 mg IgG) and it can be re-used. On the other hand, non-specific adsorption to the solid phase is greater with Sepharose than with the bacteria (Goding, 1978). Conditions for elution of complexes bound to protein A are discussed in section 6.

As free IgG is bound by protein A it must be removed before attempting to adsorb complexes on protein A Sepharose. Gel filtration followed by adsorption to protein A is preferred to precipitation with PEG or ammonium sulphate (Heimer et al., 1979) although various methods may be combined (Chenais et al., 1977; Tucker et al., 1978; Kilpatrick and Virella, 1980). IgM and polymeric IgA are not removed by initial gel filtration, however, and some may be adsorbed to protein A Sepharose. Contamination with these proteins might be reduced by eluting the complexes with buffers at, rather than below, pH 4 (Vidal and Conde, 1980). Other instances of unwanted binding to protein A may occur. For example, polyclonal IgE interacts with protein A through its $F(ab')_2$ fragment although not when an excess of IgG is present (Inganas et al., 1980) but, of course, serum usually contains only ng amounts of IgE.

Other serum components already bound in the immune complexes could interfere with the binding of IC to protein A. Apparently rheumatoid factors do not interfere because the addition of human serum with a high titre of RF to preformed IC containing rabbit antibodies did not prevent these complexes from binding to protein A coated *S. aureus* (Natali et al., 1980). However, there is some evidence that complement components may interfere. Preformed IC, solubilized with human C and mixed with anti-C3 or anti-C4, were then precipitated with protein A which bound to the antibody specific for C3 or C4 but surprisingly not to the antibodies in the initial complex (Scharfstein et al., 1979) presumably because the C components caused steric hindrance (Nussenzweig, 1980). This may be important when protein A is used to isolate IC with bound endogenous complement from clinical material.

(b) Binding to receptors on cell membranes

Raji cells. Many B type lymphoblastoid cell lines bind immune complexes and some have been used in the detection of IC (Theofilopoulos and Dixon, 1979). One of these, Raji cells, was selected because they have few or low affinity receptors for IgG Fc thus binding little 7S IgG; they were used to isolate complexes from which antigens were then separated and antisera raised against them (Theofilopoulos et al., 1978). Using model systems these authors showed that IC preformed either in vitro or in vivo could be adsorbed to C3b and other C receptors on the Raji cell surface in sufficient

amounts to be radiolabelled, eluted with acid buffers and dissociated into the constituent antigen and antibody on sucrose density gradients or by SDS-PAGE. About 30 μg antigen (BSA) bound to (Theofilopoulos et al., 1978) and could be eluted from (Tucker et al., 1978) 1×10^8 Raji cells. However, Tucker et al. preferred isolation by protein A because they found that culturing sufficient Raji cells was cumbersome, that small IC are inefficient at fixing complement and that a number of other proteins were eluted from the cell surface thus hindering antigen identification. For example, antibodies to Raji cell surface antigens and some nuclear antigens such as DNA and RNP may be present in sera of patients with connective tissue diseases and will bind to the Raji cell surface (Horsfall et al., 1981). As a result this method has not been widely used to isolate IC and to our knowledge neither have other cell types, e.g., platelets. However, it was possible to identify antigen while immune complexes were still bound to the Raji cell membrane by means of specific fluorescent antibodies (Amoroso et al., 1980). Thus where the antigenic constituent of the IC is known or suspected this approach could be useful.

(c) Binding to complement components and conglutinin

Agnello et al. (1970) showed that IC could be precipitated directly by C1q after diffusion of both reagents in agarose gel and described the optimal conditions of pH, ionic strength, concentration of EDTA and temperature for precipitation. More recently, solid phase radioassays have been developed to detect IC which bind C1q (Hay et al., 1976) and bovine conglutinin (Casali et al., 1977). Complement-reacting IC bind to C1q through the Fc part of the complexed antibody and to conglutinin through inactivated C3b fixed to the IC. These assays are used extensively to detect IC in clinical material and IC might also be analysed by means of radiolabelled antibody to putative antigens or to different Ig isotypes (Hay et al., 1976).

Direct precipitation by C1q in free solution was used to isolate IC containing IgG and IgM rheumatoid factors from rheumatoid sera and joint fluids (Winchester et al., 1970), but Chenais et al. (1977) considered that the large amounts of purified C1q required to precipitate IC in free solution limited the usefulness of the method. Components of complement may be immobilised, however, on a column of sufficient size to adsorb and elute IC for characterisation. C1q has been coupled to Sepharose (Svehag and Burger, 1976) and both C1q and conglutinin were coupled to polymethylmetacrylate (PMMA) beads by Casali and Lambert (1979). After precipitation with PEG, enough preformed soluble IC could be adsorbed and eluted from C1q coated beads to identify the antigens and antibodies, C1q, C1r, C1s and C3. Not all IC bind C1q: only those containing Igs which activate and assemble classical pathway components thus excluding IgG4, IgA and IgE. The molecular composition or minimum size of soluble IC which can activate complement is still debated but IC consisting of IgG antibodies seem to require a lattice of more than 3 antibody molecules (Mannik, 1980; Porter, 1980).

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The capacity of IC to bind C1q parallels their capacity to activate the complement sequence unless the antigen itself has a high affinity for C1q.

Some IC which can activate the classical pathway may not be bound because they are already saturated with endogenous C1q (Chenais et al., 1977). The addition of EDTA to the sample before isolation with solid phase C1q was suggested by Zubler and Lambert (1977) to prevent extrinsic C1q from binding to intrinsic C1qrs (i.e., C1) rather than to the relevant antigen-antibody complex. Lurhuma et al. (1976) claimed that EDTA released C1q from this intrinsic C1 complex which then competed with the extrinsic C1q for the antigen-antibody complex. This, however, does not happen if the concentration of EDTA does not exceed 20 mM (Shepherd, pers. comm.). Casali and Lambert (1979) isolated complexes on solid phase C1q both with and without the addition of 20 mM EDTA but did not comment on the results. The binding of IC to C1q is much greater at ionic strengths lower than physiological; Svehag (1975) bound 3 times more poliovirus anti-poliovirus at $\mu = 0.075$ than at $\mu = 0.15$. Under these conditions, however, non-specific adsorption of C1q to the solid phase becomes a problem as does non-specific binding to other materials (Agnello et al., 1971) which include DNA and other polyanions (Woodroffe et al., 1977), endotoxin lipopolysaccharides (Cooper and Morrison, 1978), and some viruses (Cooper et al., 1976). However, Casali and Lambert (1979) apparently did not identify any such contaminants in IC isolated with solid phase C1q possibly because any contaminants were lost during the preliminary PEG precipitation step.

Conglutinin bound to solid phase reacts with IC containing C3bi and therefore can be used to detect and isolate IC which activate C by both classical and alternative pathways, i.e. IC containing IgG4, IgA and IgE as well as IgM and the other IgG isotypes. Conglutinin is a normal serum protein found in many bovine species which will bind to human inactivated C3b in the presence of Ca^{2+}. The method for its preparation is described by Lachmann and Hobart (1978). Isolation with conglutinin is highly specific; less than 0.5% of 7S monomer IgG is retained (Casali and Lambert, 1979). After a preliminary PEG precipitation and concentration step, recovery of IC varied between 40% (Casali and Lambert, 1979) and 7—10% (Male, pers. comm.) but the greatest recovery was of the largest IC (>19S). C3b must be bound by the IC before they can be isolated and, if no endogenous complement has been bound, fresh normal human serum is added (Czop and Nussenzweig, 1976). C3bi in complexes is liable to be degraded by proteolytic enzymes in biological fluids. Therefore the addition of enzyme inhibitors such as phenyl methyl sulphonyl fluoride (PMSF: final concentration of 0.5 mM) (Lachmann and Hobart, 1978) to the sample is recommended. Enzyme inhibitors might also be added to the eluates since Male (pers. comm.) found that further breakdown of C components occurred before analysis by two-dimensional SDS-PAGE. Analysis of conglutinin-binding complexes from synovial fluids of rheumatoid patients showed that, of the 16 different components detected, IgM, IgG and IgA, C1q, C1r, C1s,

C3c, C3d, C3bi and Bb could be identified in the isolated complexes: 6 other components in trace amounts were not identified (Male, pers. comm.). Casali and Lambert (1979) recovered rheumatoid factor activity and antibody to leishmania antigens as well as the other Igs and C components from conglutinin-binding complexes of a patient with Kala-azar.

Recovery is low by the conglutinin method because only the sub-population of complexes carrying undegraded C3bi is recovered but if the IC containing material is concentrated beforehand a large amount can be processed depending on the size of the conglutinin column. Another disadvantage was recently reported by Pereira et al. (1980), who found that low molecular weight material from clinical samples, after fractionation on sucrose density gradients and identified as 7—8S, bound to solid phase conglutinin. The same fractions were also bound by solid phase anti-C3 (see section 5(d)). Pereira et al. (1980) concluded that this 7—8S material was IgG antibody which was no longer bound to Ag but retained C3bi. This explanation is supported by Takahashi et al. (1977) who showed that model AgAb complexes after solubilisation by complement spontaneously dissociated with release of Ag but leaving Ab and C3 peptides.

(d) Binding to anti-immunoglobulins and anti-C3

Immunoprecipitation or binding to solid phase anti-Ig antibodies can be used to isolate IC but will also isolate unbound Ig of the isotype in question. Even so, this is a useful preliminary purification step for isotypes other than IgG, for example, for IC containing IgM rheumatoid factor (Jones et al., 1980); the concentration of IgG in serum is too high for a significant recovery of complexes containing IgG.

Rheumatoid factors (IgM anti-IgG) bind to aggregated IgG or to IgG in IC in preference to 7S monomer IgG. Therefore in theory, RFs should react with IC in whole serum without interference by normal IgG. However, they frequently have some activity for monomeric IgG and this will be a contaminant in isolated complexes (Vaughan, 1956; Gilead and Sulitzeanu, 1979). There are RFs with specificities for all the subclasses of IgG and sometimes for IgA or IgE. They bind to IC within a wide range of AgAb ratios and therefore will bind both C fixing and non-C fixing IgG complexes.

Rheumatoid factors against IgG and IgA occurring naturally in man (Lurhuma et al., 1976) or raised in rabbits (Levinsky and Soothill, 1977) have been used to detect IC but in these assays the IC act as inhibitors in the agglutination of particles coated with $(IgG)_n$. Therefore scaled-up versions are unsuitable for isolated procedures although inhibition assays can be used to identify the antibody isotopy in IC (e.g., Kauffmann et al., 1981). Low affinity rabbit IgM anti-Fc has been used to bind IgG complexes after their precipitation by 2.5% PEG (Harkiss and Brown, 1980) but attempts to use solid phase IgM anti-Fcγ to isolate such complexes (André et al., 1975) were not successful because yields were low and the binding capacity of the column was rapidly lost (Chenais et al., 1977). Recently an isolation method

has been devised using human polyclonal RF which was bound to plastic tubes through a glutaraldehyde-treated IgG coating. The RF bound model complexes composed of [^{125}I]tetanus toxoid-human antitoxoid antibodies which, after elution, were contaminated with IgM (presumably eluted RF) and non-antibody IgG but contained enough antigen to be detected by autoradiography (Gilead and Sulitzeanu, 1979).

Some rheumatoid factors will precipitate IC in free solution without precipitation of 7S IgG and some purified monoclonal RFs will precipitate smaller sized complexes than those precipitated with polyclonal RFs or those which bind C1q (Winchester et al., 1971). A monoclonal RF reagent was used to isolate complexes of IgG anti-IgG from rheumatoid synovial fluids (Winchester, 1975). However, naturally occurring monoclonal RFs are rare and precipitation in free solution is uneconomical; therefore although they are used for detection they have not been widely used for isolation of IC. Production of monoclonal antibodies to various human Ig determinants (Jefferis et al., 1980) should eventually make monoclonal RF reagents available in amounts sufficient to isolate IgG containing complexes.

The anti-antibody described by Kano et al. (1978) is a naturally occurring human IgM antibody specific for the $F(ab')_2$ of IgG antibodies but only when complexed with antigen; thus it will not bind monomeric nor aggregated IgG. The anti-antibody is extremely rare, being found in useful titres (e.g., 1:80) in only about 1 in 500 blood donors. It is detected by agglutination of group O, R_1r erythrocytes sensitised with anti-Rh (C, D) antibodies but only of the Ripley type, and thus agglutination may be due to anti-idiotypic interaction. Antibodies with specificity for possible conformational determinants present only on $F(ab')_2$ combined with antigen would be ideal reagents for the isolation of IC but, although this approach has theoretical appeal, it has little practical value at present.

Antibodies to both C3 and C4 have been used in free solution to isolate preformed complexes treated with human C, by subsequent binding of the anti-C3 or anti-C4 to protein A (see section 5(a); Scharfstein et al., 1979). Solid phase $F(ab')_2$ of anti-C3 has been used recently to detect IC and the assay could be developed to characterise the antigen in IC (Pereira et al., 1980) or to isolate the complexes (Jones, unpublished). The specificity of the antiserum could be important because antibodies to C3 fragments which do not remain bound to IC would not function in the test. The anti-serum used by Pereira et al. (1980) had specificities for native human C3 and its fragments C3b, C3c and C3d whereas antisera to C3 available commercially are mainly anti-C3c (Holt, pers. comm.; Seward Laboratories, pers. comm.). Solid phase anti-C3 should react with IC which fix C activated by both classical and alternative pathways but, unlike conglutinin, it is not restricted to binding the rather labile component C3bi; thus it should have considerable advantages for isolating IC. However, as already mentioned (see section 5(c)) Pereira et al. (1980) found that the anti-C3 reagent primarily bound a 7–8S IgG fraction in many clinical samples. This type of 'complex' almost cer-

tainly lacks antigen and could be confusing in procedures designed to identify the antigen.

(e) Binding to antigen-specific antibody

Antigen-specific antibodies have not, to our knowledge, been used to isolate the IC found in infections although complexes are known to be present in the circulation, e.g., in onchocerciasis (Paganelli et al., 1980), but they are used for the identification of the constituent antigen after isolation by other methods. For example, in acute viral hepatitis associated with 'serum sickness-like' syndrome, cryoprecipitates were isolated and redissolved in PBS but were not dissociated further before HBsAg was identified in the complexes (Wands et al., 1975). In both acute and chronic hepatitis, circulating IC were concentrated by 3.5% PEG precipitation; antibody was destroyed or modified (by heating to 56°C for 1 h or by acid treatment (0.2 M glycine HCl pH 2.8) or with proteolytic enzymes) before detection of HBsAg (Carella et al., 1977; Anh-Tuan and Novák, 1980). In several virus diseases, including hepatitis (Pernice et al., 1979a) influenza A and rubella (Pernice et al., 1979b) antigen-specific antibodies have been used in an enzyme-linked immunoassay (Pernice and Sedlacek, 1979) to detect IC and thus identify the constituent antigen. For detection, the antigen-specific antibodies are bound to solid phase and therefore the method could be adapted for preparative use. The assay mainly detects those IC in slight antigen excess although specific antibody can recognise hepatitis antigen in undissociated cryoglobulins which are usually at equivalence or in slight antibody excess (Levo et al., 1977). Free antigen can block the immunoassay (Pernice et al., 1979a) and ideally should be removed before isolation of the complexes.

(6) REAGENTS FOR DISSOCIATION AND ELUTION OF IMMUNE COMPLEXES

The choice of eluant (see Table 2) depends on several factors, which include (1) the affinity of the ligands used to isolate IC; more powerful reagents are needed to elute from high affinity ligands, e.g., acid or alkaline buffers from protein A and anti-Ig columns, and milder eluants for lower affinity ligands, e.g., 0.02 M EDTA pH 7.4 for solid phase conglutinin. Another factor (2) is whether intact IC are required, or (3) whether optimum recovery of the antibody or the antigen is more important. In the latter case it would be acceptable for antibody activity to be destroyed by heating or phenol if the antigen is resistant to these treatments. Further considerations are (4) whether the biological function of the antigen or antibody has to be retained for further analysis and (5) whether the ligand is to be re-used.

The efficacy of a particular eluant is usually measured in a model system using preformed IC incorporating an ^{125}I-labelled antigen and the comparison of different eluting agents is based on the percentage of radioactivity recovered in the eluate. Even with this simple system estimates of efficiency

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ur knowledge, been used to complexes are known to be sis (Paganelli et al., 1980), but stituent antigen after isolation iral hepatitis associated with ates were isolated and redis- r before HBsAg was identified h acute and chronic hepatitis, G precipitation; antibody was for 1 h or by acid treatment tic enzymes) before detection l Novák, 1980). In several virus 979a) influenza A and rubella bodies have been used in an llacek, 1979) to detect IC and detection, the antigen-specific herefore the method could be inly detects those IC in slight n recognise hepatitis antigen in lly at equivalence or in slight ntigen can block the immuno- ıld be removed before isolation

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TABLE 2

Examples of eluants used to desorb IC from various ligands.

Eluants	Ligands	Comments
Acid		
Isotonic citrate pH 3.2	Raji cells	antigen was recovered in immunogenic form (Theofilopoulos et al., 1978)
1 M acetic acid 0.1 M glycine HCl pH 2.4 citrate phosphate pH 3.0 0.2 M glycine HCl pH 3.0	protein A Sepharose	antigenicity (Chenais et al., 1977) and antibody (Tucker et al., 1978) function (Kilpatrick and Virella, 1980) recovered
Alkali		
1 M ammonia	anti-human μ	>80% recovery of IgM RF and IgG RF (Jones et al., 1980)
Chaotropic agent		
3 M NaSCN	protein A Sepharose	antigenicity retained; 51—72% of [^{125}I]antigen recovered (Heimer et al., 1979)
Chelating agents		
0.02 M EDTA pH 7.4 in VBS 0.02 M EDTA + 0.35 M NaCl in VBS	conglutinin C1q	mild eluants: 40—60% of total antigen recovered; antibody function recovered (Casali and Lambert, 1979)
Others		
2.5 M $MgCl_2$ pH 7.0	protein A bacteria	antigenicity retained; no elution of bacterial components (Natali et al., 1980)
0.1 M lithium diiodosalicylate	protein A bacteria	antigenicity retained but antibody function reduced (MacSween and Eastwood, 1978)
0.2 M 1,4-diaminobutane + 0.075 M Tris HCl pH 8.1	C1q	damage to antigen (EAV virions) (Svehag and Burger, 1976)
0.005 M 2,5-diaminotoluolsulphate + 0.075 M Tris HCl pH 8.2	C1q	'sarcoidosis antigen' recovered in immunogenic form (Glikmann et al., 1979).

of an eluting agent vary from one laboratory to another. For example, Tucker et al. (1978) found that 0.1 M glycine HCl pH 2.4 released 54% of [^{125}I]-BSA from a preformed complex bound to protein A Sepharose, whereas Heimer et al. (1979) recovered only 10—17% of [^{125}I]HSA from a preformed complex bound to the same ligand and using the same buffer (pH 2.4 glycine). Thus the selection of eluants is often empirical.

Antibody function or antigenicity is frequently demonstrated in the eluates but quantitative recovery of these properties is difficult and rarely done. For example, all antibodies and most antigens retain some of their reactivity after treatment in acid buffers (e.g., Tucker et al., 1978) although some rheumatoid factor activity may be lost after exposure to acid; a good recovery of both IgG and IgM RF activity was achieved after alkaline elution from solid phase anti-μ (Jones et al., 1980) but much less was recovered with 0.1 M glycine HCl pH 2.8 (unpublished).

There have been many studies on the effectiveness of different reagents in dissociating AgAb complexes and eluting antibody from immunoadsorbents (e.g., Ternynck and Avrameas, 1971) and more recently Bartolotti (1977) and Woodroffe and Wilson (1977) compared the efficiency of reagents in eluting IC from kidney tissues. These latter reports provide useful information on properties of eluants such as that the acid buffer 0.02 M citrate pH 3.2 best preserved antibody binding activity, whereas it impaired the C fixing activity of the antibody compared with chaotropic ions (potassium iodide or potassium isothiocyanate; 2.5 M, pH 7.0) (Woodroffe and Wilson, 1977).

Desorption by electrophoresis in various buffers (Tris, citrate and glycine, 0.01—0.05 M and within the range pH 5.1—9.6) was used to remove rabbit antibodies from solid phase concanavalin A and human IgG (these ligands were the antigens) and to desorb IgG from protein A (Morgan et al., 1978). No information on desorption of IC is available but, because of its mildness and apparently excellent recoveries, this potentially useful method might have considerable advantages over conventional acid elution. Isoelectric focusing also has potential for separation of IC and recovery of antigen and antibody (Maidment et al., 1980). Complexes of BSA anti-BSA and of acid and alkaline phosphatases and antibody were completely separated while retaining their immunological characteristics and the hydrolytic activity of the enzymes remained intact.

High ionic strength buffers (0.4 M NaCl, 0.1 M Na_2H_2 EDTA pH 7.4, conductance 45 mmho/cm) are not only mild and efficient eluants for solid phase C1q but also prevent binding of anionic substances to the C1q and adsorption of protein to the solid phase (Gabriel and Agnello, 1977). Even veronal-buffered saline (conductivity about 10 mmho/cm at 4°C) will prevent adsorption of monomeric IgG to the solid phase (Casali and Lambert, 1979). Nardella and Mannik (1978) considered that non-immunospecific binding of IgG to agarose was due primarily to protein-protein interactions which can be measured by using buffers at twice physiological ionicity and alkaline pH.

In conclusion, with all elution techniques the least avid antibodies are

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(7) DETECTION OF ANTIGEN AND ANTIBODY

While it is easy to demonstrate the presence of antigen and/or antibody in model complexes, attempts to identify suspected constituents in clinical material pose considerable problems even when the same methods are used. Furthermore detection of antigen by recognition of its antigenic sites and detection of antibody by its capacity to combine with antigen could be hindered if antigen and antibody are not separated after elution and the IC is allowed to reassociate at neutral pH and isotonicity. Nevertheless some epitopes can be detected in undissociated IC, for example, specific fluorescein-labelled antisera can detect antigen in complexes bound to cell surfaces before the IC are eluted, e.g., from Raji cells (Theofilopoulos et al., 1976; Amoroso et al., 1980). Damaged virions were seen by electron microscopy in complexes which had been preformed and then isolated by solid phase C1q without further separation (Svehag and Burger, 1976). In a preliminary report Svehag et al. (1979) used antibodies to suspected antigens, e.g., *Varicella zoster*, to identify the constituent Ag in complexes bound to solid phase C1q and in solution by re-precipitation. Digestion of complexes with enzymes specific for suspected antigens could be useful, for example, DNase which released bound DNA antibody in a patient with active SLE (Bruneau et al., 1977), RNase and perhaps collagenases.

Injection into rabbits of undissociated complexes of rabbit antibody and BSA or HSA which had been isolated from Raji cells or bound to protein A bacteria produced antibodies to BSA and HSA (Theofilopoulos et al., 1978; Natali et al., 1980). Laboratory animals have rarely been immunised with IC of unknown constitution isolated from patients' sera in order to identify the constituent antigen but two examples are the following. Winchester et al. (1970) made antisera against IgG complexes purified from rheumatoid synovial fluids but the antisera failed to reveal any antigens other than IgG. In a preliminary study Glikmann et al. (1979) immunised rabbits with complexes isolated from two sarcoidosis patients and used the antisera to detect and partly characterise an antigen with post-albumin electrophoretic mobility and molecular size of >19S in 7 patients.

Immunisation of laboratory animals with antibodies dissociated from IC might be used to detect possible idiotypes in the hope of finding an association between a particular idiotype and a disease. For example, a common idiotype was detected in both the cerebrospinal fluid and serum of a patient with multiple sclerosis (Baird et al., 1980). There are examples of cross idiotypes relating to the specificity of the antibody, such as cold agglutinins (Williams, 1971). Also Agnello et al. (1980) identified an idiotype in a

human monoclonal RF with reactivity to DNA-histone and found the same idiotype in a polyclonal RF with reactivity to DNA-histone. However, raising anti-idiotypes and interpreting their specificities present considerable problems which have been reviewed by Sogn et al. (1977) and Davie (1980). It might be simpler to recover the antibody component from IC of unknown constitution and test it against a battery of likely antigens; this approach has been successful in determining the antibody specificity of many mouse myelomas (Potter, 1971).

The constituents of isolated IC have also been analysed by 1- and 2-dimensional electrophoresis in agarose and/or polyacrylamide gels with sodium dodecyl sulphate (Tucker et al., 1978; Casali and Lambert, 1979; Male and Roitt, 1979). Protein staining after SDS-PAGE revealed a 79 000 m.wt. component in complexes isolated from rats bearing tumours induced by a Gross leukaemia virus; this putative antigen reacted with antiserum raised against the virus envelope glycoprotein gp 70 (Tucker et al., 1978). Immunoprecipitation or the binding of radiolabelled specific antibody to identify antigens in SDS-PAGE has recently been reviewed by Anderton and Thorpe (1980) who point out that SDS is a strongly anionic detergent which destroys some antigens but not others. Proteins separated by electrophoresis may be transferred from gels to cellulose papers for identification by binding of radiolabelled antigen or antibody, for example: (1) to detect common antigenic determinants in related antigens (e.g. in histones from different species and in the enzyme dihydrofolate reductase from mammals and bacteria) using an antiserum in the overlay, (2) to identify glycoproteins using lectins in the overlay, and (3) to analyse the isoelectric focusing spectra of antibodies (e.g. to β-galactosidase) using the antigen in the overlay (Erlich et al., 1979). The technique should have wide application in the analysis of IC; two recent examples are the following. Male et al. (1980) radiolabelled the IC concentrated from synovial fluids of patients with rheumatoid arthritis and after 2-dimensional SDS-PAGE, identified the main constituents, namely, Igs, C components, fibrinogen, serum albumin and α_2-macroglobulin, by immunoprecipitation with specific antisera. Although trace amounts of other unidentified components were detected by autoradiography, the authors concluded that autologous IgG was probably the only antigen in these complexes. Heimer et al. (1979) tried another modification to identify the antigens in IC isolated from patients with SLE. After electrophoresis of the isolated IC in SDS-PAGE, the individual patients' sera and normal sera were used as antibody overlays; the antibody was visualised with [^{125}I]protein A and autoradiography. Six unidentified antigens with molecular weights below 42 000 daltons were seen; they were not exclusive to SLE because they were revealed with normal sera in the overlay but were seen more distinctly when SLE sera were used.

The analysis of IC is an area in which monoclonal antibodies formed by rat or mouse hybridomas have enormous potential. Three recent reviews (Goding, 1980; McMichael and Bastin, 1980; Staines and Lew, 1980) stress

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ponents were detected by auto-
ıtologous IgG was probably the
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30; Staines and Lew, 1980) stress

the practical aspects of this technology as it might apply to the analysis of IC and in clinical situations. An important advantage is that after immunisation with a heterogeneous antigenic mixture such as IC of unknown constitution, there is a reasonable prospect that one of the many resulting antibodies will be specific for a minor constituent, in this instance the antigen in the IC. Screening procedures to identify and discard hybrids secreting antibodies to unwanted components, namely to constant region Ig determinants, RFs and C components, should present no problems and the remainder, namely those to unusual or unique determinants, might have specificity for the antigen (or the idiotype) in the complexes. Antibodies to suspected antigens could be used in overlays, for example to characterise constituent antigens after SDS-PAGE analysis of the isolated IC (Heimer et al., 1979; cf., above) or in highly sensitive enzyme immunoassay techniques (e.g., Shalev et al., 1980) with a panel of known antigens. These antibodies might also be used to extract the suspected antigens from IC by affinity chromatography in amounts sufficient for analysis. Furthermore a few human-human hybridomas have now been made. For example, one human hybridoma which secretes anti-measles virus antibodies was made by fusing peripheral blood lymphocytes from a patient with subacute sclerosing panencephalitis with a cell line derived from a patient suffering from multiple myeloma (Croce et al., 1980). By fusing cells from patients suffering from autoimmune or 'immune complex' diseases with one of the fusable cell lines, antiidiotypes might be formed.

(8) DISCUSSION AND CONCLUSIONS

The diseases in which IC have been detected, although not necessarily shown to contribute to the pathology of the disease process, have been listed and discussed by Theofilopoulos and Dixon (1979). Many of these diseases are of unknown aetiology such as the autoimmune diseases, some forms of glomerular nephritis and the neoplastic diseases, and in many of these neither the isolation of circulating IC of unknown constitution nor identification of the antigens and antibody content are easily achieved. One problem is the heterogeneity of IC in the circulation, not all of which may be related to the disease under study. In SLE, circulating IC contain a variety of native or altered autologous antigens and Izui et al. (1977) found in one study that although 52% of SLE patients had circulating IC only 6% had complexes composed of DNA anti-DNA. Nevertheless, analysis of IC in diseases of unknown aetiology can be rewarding, for example, in several patients with essential mixed cryoglobulinaemia associated with arthritis, hepatitis antigen or antibody were detected in cryoprecipitates although they were not found free and uncomplexed in the serum (Levo et al., 1977). Moreover, in insulin-dependent diabetes where IC are thought to cause the microangiopathy leading to clinical complications, soluble insulin anti-insulin IC have been isolated and analysed (Kilpatrick and Virella, 1980).

The biological properties of IC, namely their rate of formation and of clearance from the circulation, their ability to form deposits in tissues and their C combining capacity, are of direct relevance in disease. Their differing biological properties are determined by their physicochemical characteristics. In addition to antigen and antibody content, IC differ in Ig isotype, molecular size and composition, ratio of Ag to Ab, affinity of the Ab and their ability to interact with RFs and conglutinin. This probably accounts for the large discrepancies in results obtained with different methods for detecting IC. The same applies to methods for isolation of IC; it would be unreasonable to expect any one method to extract in a single step all the complexes in a sample.

With the isolation techniques available at present the choice is either to concentrate and crudely purify the sample so that most complexes would be recovered but would be heavily contaminated by other serum proteins e.g. gel filtration or PEG precipitation, or to use more selective isolation methods, e.g., solid phase C1q or conglutinin which isolate a smaller proportion of the complexes in the sample, although the complexes will still be contaminated by C components and possibly RFs. In practice these two approaches are often combined. One promising isolation technique for the future could be solid phase anti-C3 of the appropriate specificity which should isolate the complexes which bind C3 activated by both classical and alternative pathways.

Another attractive possibility is the production by mouse or rat hybridomas of monoclonal antibodies for the isolation of IC. An antibody with specificity for altered Fcγ (or Fcα) might mimic RFs and bind IC or aggregated IgG but not monomeric Ig. Monoclonal Abs directed to an epitope on an antibody $F(ab')_2$ when it is complexed with antigen, if indeed such an epitope exists, would mimic the anti-antibody reported by Kano et al. (1978). Even these antibodies, however, will inevitably isolate some complexes with bound C and RF.

In this review (perhaps written prematurely) we have assessed early and often preliminary experiments designed to isolate and characterise IC of unknown constitution. But, because of the potential importance of IC in the pathogenesis of diseases, inevitably this area of research will expand. We have emphasized, perhaps unduly, the problems and pitfalls inherent in the techniques tried so far, namely the difficulty of isolating IC of sufficient purity without sacrificing a large part of the circulating IC population, the high proportion of constituents other than antigen and antibody usually bound in IC, and the difficulties in antigen identification and in discovering antibody specificity. Nonetheless, other areas of research have appeared equally daunting at first, and new technology, particularly in the fields of hybridoma monoclonal antibodies and high resolution electrophoresis, should give fresh answers in an important field.

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NOTE ADDED IN PROOF

Svehag et al. (1979) have now published in detail their methods for attempted identification of antigens in IC (Husby, Svehag, Nielsen, Høiby and Schiøtz, 1981, Acta Pathol. Microbiol. Scand. Sect. C 89, 155) and conclude that the most useful is a double PEG precipitation-immunoradiometric assay in which PEG insoluble IC are reprecipitated in the presence of [^{125}I]-Ab to suspected Ags. Gilead, Gazitt and Sulitzeanu (1981, J. Immunol. Methods 42, 67) have modified their method for isolation of IC and identification of the constituent Ag using RF as immunoadsorbent. They now treat samples with 0.02 M EDTA pH 7.2 to dissociate C components from the IC and, following IC dissociation at acid pH, remove HSA and Ig by precipitation with the corresponding Abs; in this way two putative Ag components of IC in an ovarian cancer effusion were identified. Maidment et al. (1980) have now applied their technique using preparative isoelectric focusing to recover immunologically reactive Abs and putative Ags from breast cancer IC (Maidment, Papsidero, Nemoto and Chu, 1981, Cancer Res. 41, 795). Heimer, Glick and Abruzzo (1981, Scand. J. Immunol. 13, 441) present useful modifications of their previous procedure (1979) for detection of Ags in IC.

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Antibodies

A LABORATORY MANUAL

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Cold Spring Harbor Laboratory
1988

the damage of the periventricular structures. An additional advantage of this method is the simplicity and unexpensiveness of the technical tools as compared to the methods using pressure transducers and recording devices[3,4].

1 This work was done under a contract with the Association of Finnish Life Insurance Companies.

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Immunogenicity of agarose-immobilized immune complexes[1]

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Summary. A novel method is described for production of heterologous antisera to a specific tumor-associated murine antigen by immunization with agarose-trapped immune complexes.

Preparation of high titer heterologous antisera to specific tumor-associated antigens is complicated by the use of crude tissue extracts as immunogen in which the relative concentration of the antigen is quite low. Where the tumor-associated antigen is present in moderate quantities, reasonable quality antisera can be obtained by immunization with unfractionated tissue extracts and appropriate serum absorptions. Such antisera have been used to precipitate antigen from solution and the immune complexes, in turn, employed as immunogens to elicit the formation of a more highly specific antiserum[2]. The limiting factors, however, of sufficiently absorbed precipitating antibody, low concentrations of specific antigen in crude tissue extracts and difficulties in the efficient processing of minute precipitates combine to make effective immunization with solution-precipitated immune complexes a prodigious task.

A simple method for the preparation of high titer antisera to a specific tumor-associated antigen, murine γ-FA[3-5], is described. This procedure should be applicable to studies of diverse tissue antigens.

Methods and results. Immunization of rabbits with saline extracts of a mouse fibrosarcoma, antiserum absorption and subsequent identification of an antigen common to tumor, fetal and adult splenic tissue, termed γ-FA, has been described[3-5]. Radial immunodiffusion plates[6] contained 0.1 ml of absorbed anti-γ-FA serum and 2.5 ml of 1% agarose (w/v) in Beckman 3-2 buffer, pH 8.6. Antigen wells (3.2 mm in diameter) were cut into the agarose gel, filled with 7 µl of the 10,000 × g supernatant fraction of a 10^{-3} M Tris, pH 7.5, homogenate of normal adult mouse spleen and the plates incubated at 37 °C for 72 h to allow for precipitin ring formation. The agarose slabs were then dialyzed with stirring against daily 200-ml changes of phosphate-buffered saline (PBS) for 3 weeks at 4 °C in order to remove unbound protein. The use of a 1% agarose gel facilitated this removal while antigen-antibody complexes remained trapped within the gel matrix. After dialysis, sections of gel containing precipitin rings were cut out, passed several times through an 18-gauge needle and frozen at −20 °C in twice the volume of PBS. On day 1, 14 and 21, 1 ml of agarose-immobilized immune complexes was emulsified in 1 ml of complete Freund's adjuvant and inoculated s.c. into a New Zealand white rabbit (on day 21 incomplete adjuvant was substituted for complete adjuvant). 22 days later the rabbit was bled and 1-ml aliquots of the antiserum inoculated i.p. into each of several adult C57 mice for in vivo absorption. After 24 h, the mice were bled and the antibody activity of the absorbed antiserum compared with that of the original anti-γ-FA serum. Clearly, the antiserum to γ-FA-anti-γ-FA immune complexes possessed all the precipitin specificity of the original anti-γ-FA serum but at a much higher titer (table) and, unlike the original antiserum, yielded a positive indirect immunofluorescence test[7] on methanol-fixed rat hepatoma cells (figure). This antigen was previously thought to be synthesized only by in vivo propagated tumor cells[4] and the present data, therefore, provide the 1st direct evidence for production of γ-FA by transformed cells. Moreover, reten-

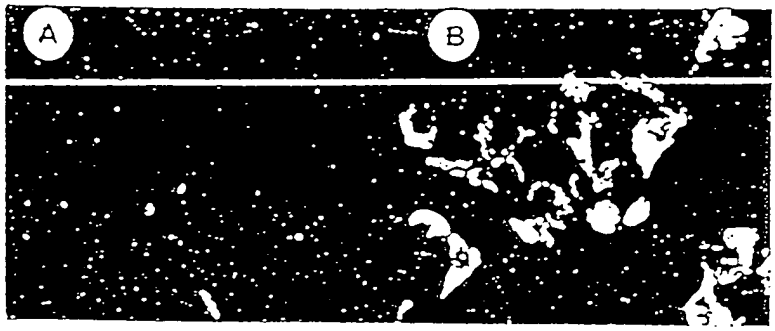

Fig. 1. Indirect immunofluorescence test of in vitro propagated rat tumor cells isolated from a transplanted hepatoma[3]. *A* Original anti-γ-FA serum (1:20); *B* anti-γ-FA immune complexes (1:60). UV light microscopy. BG12-53/44 filters, Zeiss Photoscope.

Table I. Comparative precipitin tests[a]

Test antigen	Original anti-γ-FA (undiluted)	Anti-γ-FA immune complexes (1:30)[b]
Meth A serum[c]	+	+ + +
Meth A tumor[d]	+	+ + + +
Normal serum[e]	–	–
Adult spleen[f]	+	+ + + +
Viscera[g]	–	–

[a] Hyland Immuno-Plates, pattern 'D'. Preliminary experiments indicated the precipitin line which formed upon interaction of the original anti-γ-FA serum with a saline extract of adult mouse spleen and that which formed by interaction of anti-γ-FA immune complexes with the same splenic tissue extract was one of identity. [b] Calculated dilution after in vivo absorption. [c,d] Serum and saline extract of tumor tissue obtained from a mouse bearing a transplanted 3-methylcholanthrene-induced fibrosarcoma. [e,f] Obtained from normal adult mice. [g] Individual saline extracts of adult mouse liver, kidney, brain, heart, lung, testes and small intestine pooled from several mice.

tion of antibody activity after in vivo absorption in mice (which have γ-FA-positive spleens) suggests that γ-FA is not a cell surface antigen.

Preparation of a high titer anti-γ-FA serum by immunization with agarose-trapped immune complexes will facilitate future in vitro studies of various aspects of cellular transformation. In addition, the method described should be adaptable to diverse tissue antigen systems.

1 Supported in part by a Biomedical Research Support Grant and Grant ROI-CA-20019-04 from the National Cancer Institute.
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CONGRESSUS

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Manuscripts (including all figures and tables) should be submitted in duplicate.

Text should not exceed 2–3 typewritten pages (50–60 lines). 1–2 relevant figures or tables. English summary of maximum 4 lines. Abbreviations should be properly explained. References should be numbered consecutively and be presented on a separate page. Name and address have to be placed directly under the title. Linguistically inadequate manuscripts will be returned. Manuscripts in languages other than English should be supplemented by an English translation of the title. Footnotes should be avoided.

Figures (illustrations) should be separate from the text, with the author's name on the back in soft pencil. The desired labelling should be shown on a second set of figures, which will be used as a model for inscriptions. Drawings for reproductions should be on good paper in Indian ink, photographs should be supplied as glossy positive prints. The illustrations should be at least one and a half times as large as the definitive size desired. Over-large figures can be easily damaged in the mail. Captions should be selfexplanatory, without reference to the text.

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3 ANTIBODY–ANTIGEN INTERACTIONS

The interaction of an antibody with an antigen forms the basis of al immunochemical techniques. This chapter discusses the properties o the antibody–antigen interaction and is divided into three section The first summarizes the structure of the antibody–antigen bonds, th second covers the strength of these interactions, a characteristi known as affinity, and the third presents the factors that contribute t the overall stability of immune complexes, a property called avidity

STRUCTURE OF THE ANTIBODY–ANTIGEN COMPLEX

The structure of the antibody–antigen complex has been studied b measuring the affinity of binding between an antibody and a series o related antigens, by using affinity labeling reagents, by site-directe mutagenesis of the antibody combining site, by molecular modeling and, most compellingly, by X-ray diffraction studies of antibody antigen cocrystals. Together, these techniques have delineated th region of the antibody molecule that is involved in antigen binding, th region of the antigen molecule that interacts with the antibody, and th molecular basis for antibody specificity.

The antigen binding site of an antibody is formed by the variable regions of the heavy and light chains

Affinity labeling and X-ray crystallography of immune complexe have established that the antigen binding site is formed by the heavy and light-chain variable regions (see Fig. 2.5). The two variable region are closely associated and are bound to each other by noncovalen interactions. The remainder of the heavy and light chains forms othe domains that are not involved in antigen binding (see Chapter 2). Th amino acids forming the antigen binding site are derived from both th heavy and light chains and correspond to the amino acids of th hypervariable regions determined from protein sequencing. The hy pervariable regions are known as the complementarity determinin regions (CDRs). There are six CDRs, three on each chain, and the form discrete loops anchored and oriented by the framework residue of the variable domains (Fig. 3.1).

The region of an antigen that binds to an antibody is called an epitope

The region of an antigen that interacts with an antibody is defined a an epitope. An epitope is not an intrinsic property of any particula

structure, as it is defined only by reference to the binding site of an antibody. The size of an epitope is governed by the size of the combining site. From X-ray studies of the structures of cocrystals between small antigens bound to antibodies, the size of the combining site was thought to be relatively small. The site was visualized as a cleft or pocket into which the epitope docked. Relatively few of the amino acid side chains of the CDR were in close contact with the antigen. Recently, the structures of three protein antigen–antibody complexes have been solved. These structures have changed our view of the potential size and shape of epitopes. The two antigens used in these studies were lysozyme and influenza neuraminidase. The area of these antigens in close apposition to the antibody is large, occupying approximately 500–750 $Å^2$ and involving contacts with all six CDRs. Although these studies have shown that epitopes can be much larger than originally thought, it is still clear that high-affinity antibodies can be raised to small epitopes.

Because antibodies can recognize relatively small regions of antigens, occasionally they can find similar epitopes on other molecules. This forms the molecular basis for cross-reaction. Although similar epitopes can occur on related molecules, the presence of similar epitopes does not necessarily imply a functional relationship.

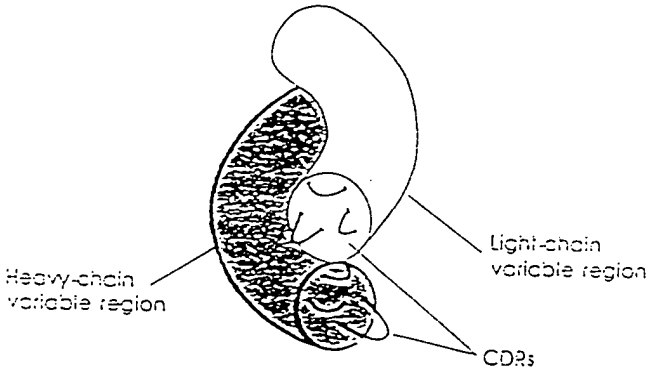

FIGURE 3.1
The six CDRs form the binding sites for antigen-antibody association.

Epitopes on protein antigens are local surface structures that can be formed by contiguous or noncontiguous amino acid sequences

In one of the lysozyme–antibody cocrystals, the amino acids of lysozyme that form the epitope come from two distant stretches of the primary sequence (residues 18–27 and residues 116–129). Though separated from each other in the primary sequence, these stretches of amino acids are adjacent on the protein surface. At the interface between the antigen and the antibody, a total of 16 amino acids of the antigen make close contacts with 17 amino acids of the antibody, the latter involving all six CDRs. The whole interface is tightly packed and excludes solvent. Strikingly, 748 $Å^2$ or 11% of the surface of lysozyme is covered by the antibody. Similar conclusions come from the study of the second lysozyme–antibody cocrystals and the neuraminidase–antibody cocrystals. Here, either three (lysozyme) or four (neuraminidase) stretches of distant primary sequence form portions of the epitope structure.

Although no cocrystals of epitopes derived from contiguous stretches of amino acids have been solved, work with antibodies raised against synthetic peptides or other small antigens has shown that these interactions exist.

Some immune complexes show no alterations in the structure of the antibody or antigen, whereas others show large conformational changes

Antibody–antigen interactions can occur either with large structural changes in the antibody or the antigen or with no detectable changes. From the structure of the first three antibody–protein antigen cocrystals, it is clear that both flexible and rigid structures can form good epitopes. In the crystal structure of one of the lysozyme–antibody complexes, no distortion of either the antigen or antibody could be detected, even at the 2.8 Å level. In sharp contrast, the crystal structure of the neuraminidase–antibody complex revealed substantial structural alterations of both the antigen and antibody. Because the crystallization process can induce structural alterations itself, it is difficult to prove that these changes are due to antibody binding. However, many other studies have shown that antibodies can induce structural changes in antigens. Good examples of this are the removal of heme from myoglobin and the activation of enzymes by antibody binding. More structural analyses are needed to determine how frequently such alterations accompany complex formation.

The antibody–antigen complex is held together by multiple, noncovalent bonds

The binding of the antibody to the antigen is entirely dependent on noncovalent interactions, and the antibody–antigen complex is in

equilibrium with the free components. The immune complex is stabilized by the combination of weak interactions that depend on the precise alignment of the antigen and antibody. These noncovalent interactions include hydrogen bonds, van der Waals forces, coulombic interactions, and hydrophobic bonds. These bonds can occur between side chains or the polypeptide backbones.

Small changes in antigen structure can affect profoundly the strength of the antibody–antigen interaction. The loss of a single hydrogen bond at the interface can reduce the strength of interaction 1000-fold. The overall interaction is a balance of many attractive and repulsive interactions at the interface. This can be demonstrated in vitro by site-directed mutagenesis. Changing the amino acid residues that form the binding site can alter the strength of an antibody–antigen interaction. This is performed elegantly in vivo by the selection of cells secreting higher-affinity antibodies. By an unknown process, the CDR residues from differentiating clones of B cells undergo extensive mutation, yielding antibodies that differ widely in the microstructure of their antigen binding sites. Cells that express antibodies with higher affinity are stimulated preferentially to divide. This process continues during the exposure and reexposure to antigen and results in a stronger and more specific antibody response (see Chapter 4).

Antibodies can bind to a wide range of chemical structures and can discriminate among related compounds

The microenvironment of the combining site can accommodate highly charged as well as hydrophobic molecules. Epitopes composed of carbohydrates, lipids, nucleic acids, amino acids, and a wide range of synthetic organic chemicals have all been identified. The repertoire of possible binding sites is enormous, and antibodies that are specific to novel compounds have been derived readily.

The specificity of antibodies has been demonstrated by a large number of experiments showing that small changes in the epitope structure can prevent antigen recognition. For example, antibodies have been isolated that will differentiate between conformations of protein antigens, detect single amino acid substitutions, or act as weak enzymes by stabilizing transition forms.

AFFINITY

Affinity is a measure of the strength of the binding of an epitope to an antibody.

The binding of antibodies to antigens is reversible, and the strength of the interaction can be described in terms of an equilibrium reaction

Antibody binding to antigen is noncovalent and reversible. The binding of antibody to antigen follows the basic thermodynamic principles of any reversible bimolecular interaction. Thus, if [Ab] = molar concentration of the unoccupied antibody binding sites, [Ag] = molar concentration of the unoccupied antigen binding sites, and [Ab–Ag] is the molar concentration of the antibody–antigen complex, the affinity constant K_A = [Ab–Ag]/[Ab]·[Ag]. In practical terms, affinity describes the amount of antibody–antigen complex that will be found at equilibrium.

The time taken to reach equilibrium is dependent on the rate of diffusion and does not vary from one antibody to another. However, high-affinity antibodies will bind larger amounts of antigen in a shorter period of time than low-affinity antibodies. In practice, this means that high-affinity interactions are substantially complete well before low-affinity interactions. High-affinity antibodies perform better in all immunochemical techniques. This is due not only to their higher capacity but also to the stability of the complex. For example, the half-time for dissociation of an antibody binding to a small protein antigen with high affinity is 30 min or more, while for a low-affinity antibody this time may be a few minutes or less.

The affinity of the antibody–antigen interaction varies over a wide range

The range of measured values of affinity constants for antibody–antigen binding is enormous and extends from below 10^5 mol^{-1} to above 10^{12} mol^{-1}. For comparison, the affinity of trypsin for its substrate is approximately 1.25×10^4 mol^{-1}, the affinity of λ repressor converting from monomer to dimer is 5×10^7 mol^{-1}, and the affinity of λ repressor for DNA is 10^{10} mol^{-1}. Like all equilibrium reactions, the affinity constant for antibody–antigen interactions is affected by temperature, pH, and solvent. Changes in these may increase or decrease the number of antibody–antigen complexes found at equilibrium. These alterations will change the affinity constant, either driving the reaction toward complete binding or releasing bound antigen.

The affinity of monoclonal antibodies can be determined exactly, but the affinity of polyclonal antibodies cannot. Because monoclonal antibodies are homogeneous, the exact measurement of their affinity is

possible using a range of techniques. Polyclonal sera contain complex mixtures of antibodies of different affinities, therefore the affinity of such sera cannot be exactly determined.

Although the antibody–antigen affinity in a particular environment does not vary, the extent of complex formation can be manipulated

The easiest way to control the extent of complex formation is to vary the concentration of the antibody or antigen. Provided neither component is saturated, adding more antibody to a constant volume will increase the amount of antigen that is bound. Similarly, adding more antigen will increase the bound antibody. With suitably high affinities, the addition of excess antibody can be used to bind essentially all of the available antigen, but with low-affinity antibodies a significant fraction of the antigen will remain free. Increasing the reaction volume, thus lowering the concentration of both antibody and antigen, will decrease the amount of complex. The amount of complex decreases approximately with the square of the volume, when neither component is saturated. For low-affinity antibodies, reaction volume will greatly influence the amount of complex formed.

Table 3.1 lists typical affinity values for antibodies and compares these values with the required affinities for several of the common immunochemical techniques.

TABLE 3.1
Factors Affecting the Strength of Antibody Binding

Cell Staining	1. Affinity, 10^5 mol^{-1} (weak signal) to 10^8 mol^{-1} (strong signal) 2. Possible bivalent binding 3. Possible multivalent binding to secondary reagent 4. Possible local concentration effects
Immunoprecipitation	1. Affinity, 10^7 mol^{-1} (weak signal) to 10^9 mol^{-1} (strong signal) 2. Possible polyclonal binding 3. Possible multivalent binding to secondary reagents
Immunoblotting	1. Affinity, 10^5 mol^{-1} (weak signal) to 10^8 mol^{-1} (strong signal) 2. Possible bivalent binding 3. Possible multivalent binding to secondary reagents 4. Possible local concentration effects

AVIDITY

Avidity is a measure of the overall stability of the complex between antibodies and antigens. The overall strength of an antibody–antigen interaction is governed by three major factors, the intrinsic affinity of the antibody for the epitope, the valency of the antibody and antigen, and the geometric arrangement of the interacting components. Since the avidity describes the complete reaction, this value ultimately determines the success of all immunochemical techniques.

When the antibody and antigen can form multivalent complexes, the strength of the interaction is greatly increased

Most immunochemical procedures involve multivalent interactions. Antibodies are multivalent; IgGs and most IgAs are bivalent, and IgMs are decavalent. Antigens can be multivalent either because they contain multiple copies of the same epitope, as in the case of homopolymers, or because they contain multiple epitopes recognized by different antibodies. Multivalent interactions can greatly stabilize immune complexes, rendering the reactions practically irreversible.

Multimeric interactions allow low-affinity antibodies to bind tightly. Because some techniques are more likely to allow multivalent interactions, low-affinity antibodies may work well in one technique, but not in another. When antibodies show this variation among different techniques, it is often due to differences in multivalent binding. Similarly, a cross-reaction that is undetectable in one technique may dominate the results of another assay.

It is not always easy to predict the effects of multivalency, as the reactions involve geometric arrangements that impose steric constraints. However, the effects of multivalency can be illustrated by considering several simple cases as described below: (1) where a monoclonal antibody interacts with a homopolymeric antigen (Fig. 3.2); (2) where polyclonal antibodies bind to an antigen that has multiple epitopes creating large multimeric complexes (Fig. 3.3); (3) where polyclonal antibodies bind to an antigen that has multiple epitopes creating a good target for multimeric binding by secondary reagents (Fig. 3.4); and (4) where an antibody binds to an antigen immobilized on a solid support (Fig. 3.5). There are other variations on these patterns, but they share the basic features illustrated by these examples.

Homopolymeric antigens present identical, repeating epitopes that encourage bivalent binding

When a monoclonal antibody binds to a multimeric antigen, the initial reaction is identical to the bimolecular interactions discussed above. The antibody finds the antigen by diffusion. However, the second step of the reaction links the unoccupied combining site of the antibody with an identical epitope on the same antigen molecule. This reaction is an intramolecular conversion and does not depend on diffusion. It is restrained only by conformation (Fig. 3.2). Once this molecular complex is assembled, the rate of dissociation of the individual antibody–epitope interactions is similar to the normal bimolecular complex. However, since the antigen will still be held by the other interaction,

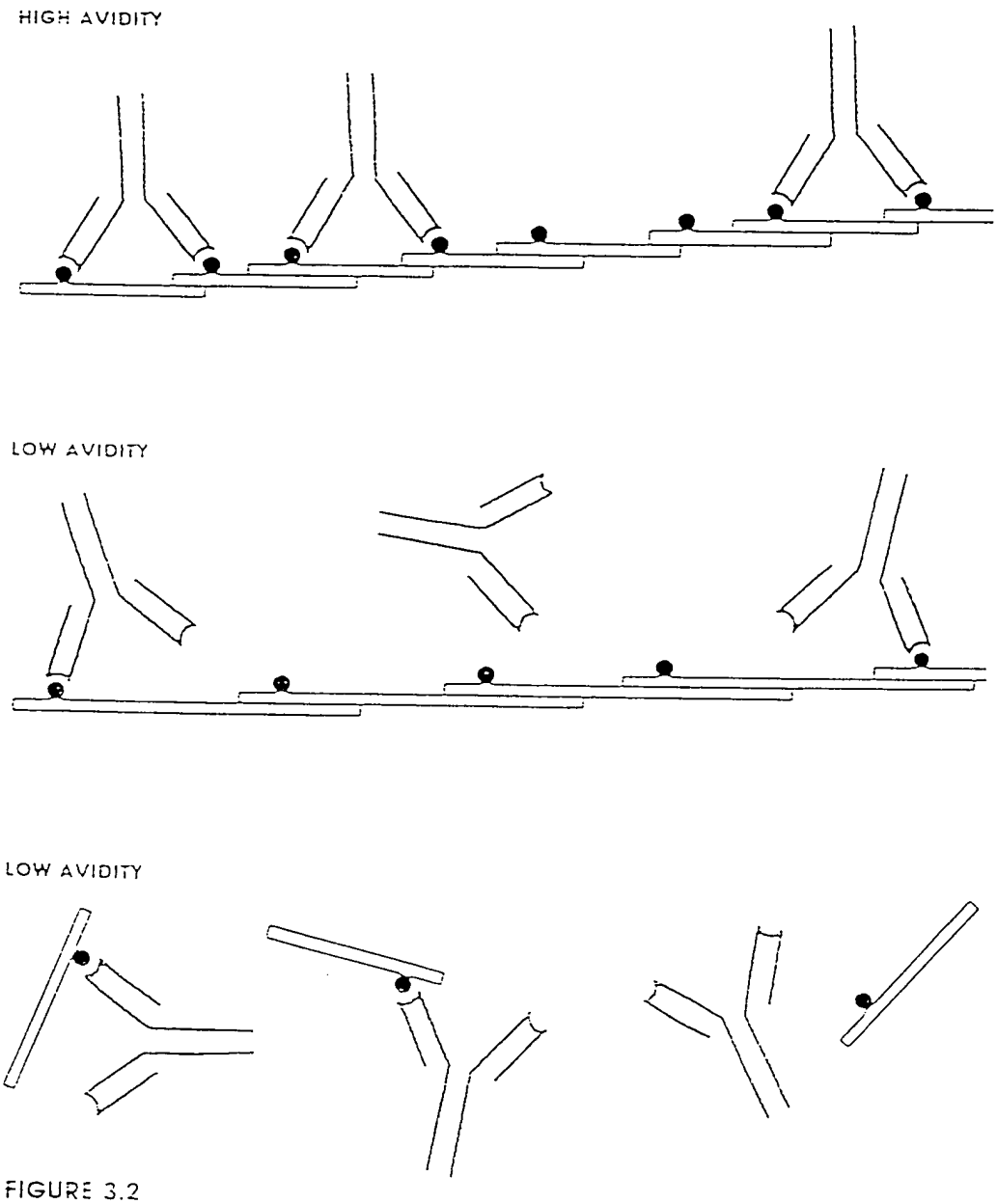

FIGURE 3.2
Bivalent binding of antibodies to polymeric antigens increases the avidity.

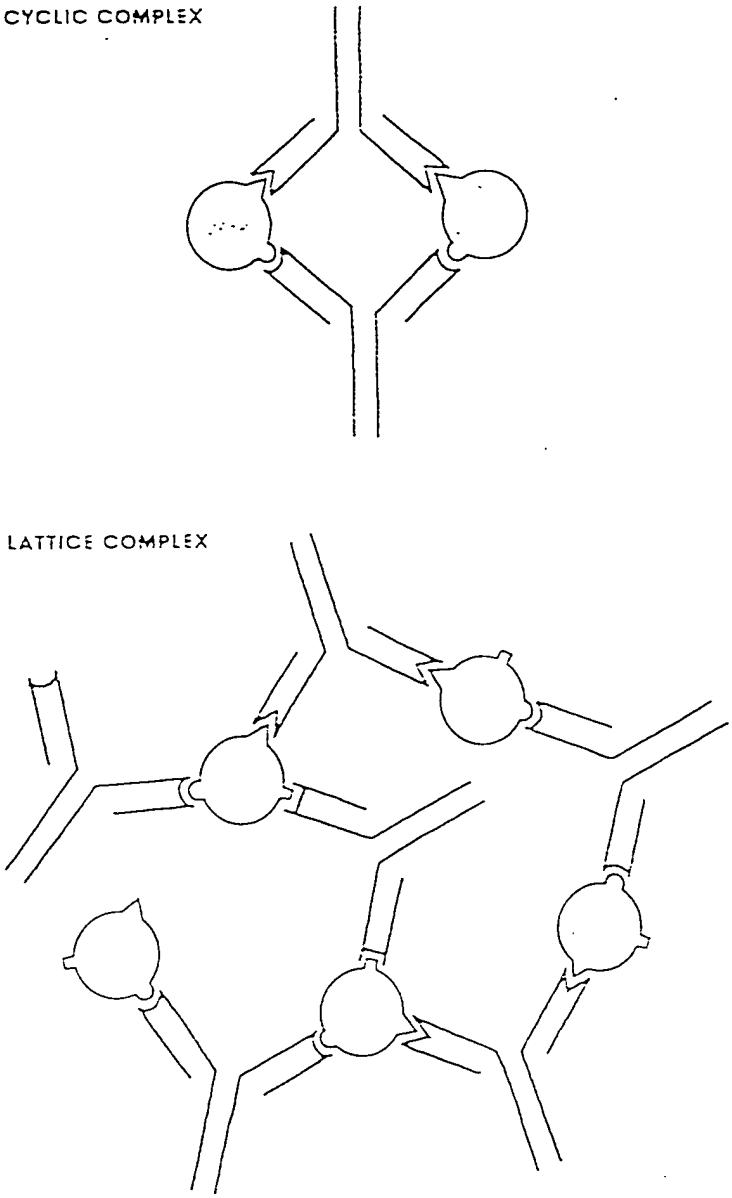

FIGURE 3.3
Polyclonal antibodies binding to multivalent antigens.

the observed rate of dissociation will be much slower, thus forming a more stable antibody–antigen complex.

This type of multimeric interaction can occur in all immunochemical techniques.

Antibodies that bind to multiple sites on an antigen can form large, stable, multimeric complexes

When an antigen with more than one epitope is mixed with polyclonal antibodies, the complexes that form can be stabilized by intermolecular bridges (Fig. 3.3). During complex formation, one antibody binds more than one antigen molecule. If these antigen molecules are linked

by the binding of a second antibody to other epitopes, a cyclic or lattice structure can be formed. The rate of dissociation of any one antibody–epitope binding is the same as for a simple interaction, but because the antigen is still held by other interactions, the overall rate of dissociation is very slow. These interactions form stable and often very large complexes. The formation of this type of complex is dependent on the relative molar ratios of the antigens and antibodies. Either used in excess will restrict the extent of cross-linking.

This type of multimeric interaction can occur in immunoprecipitation.

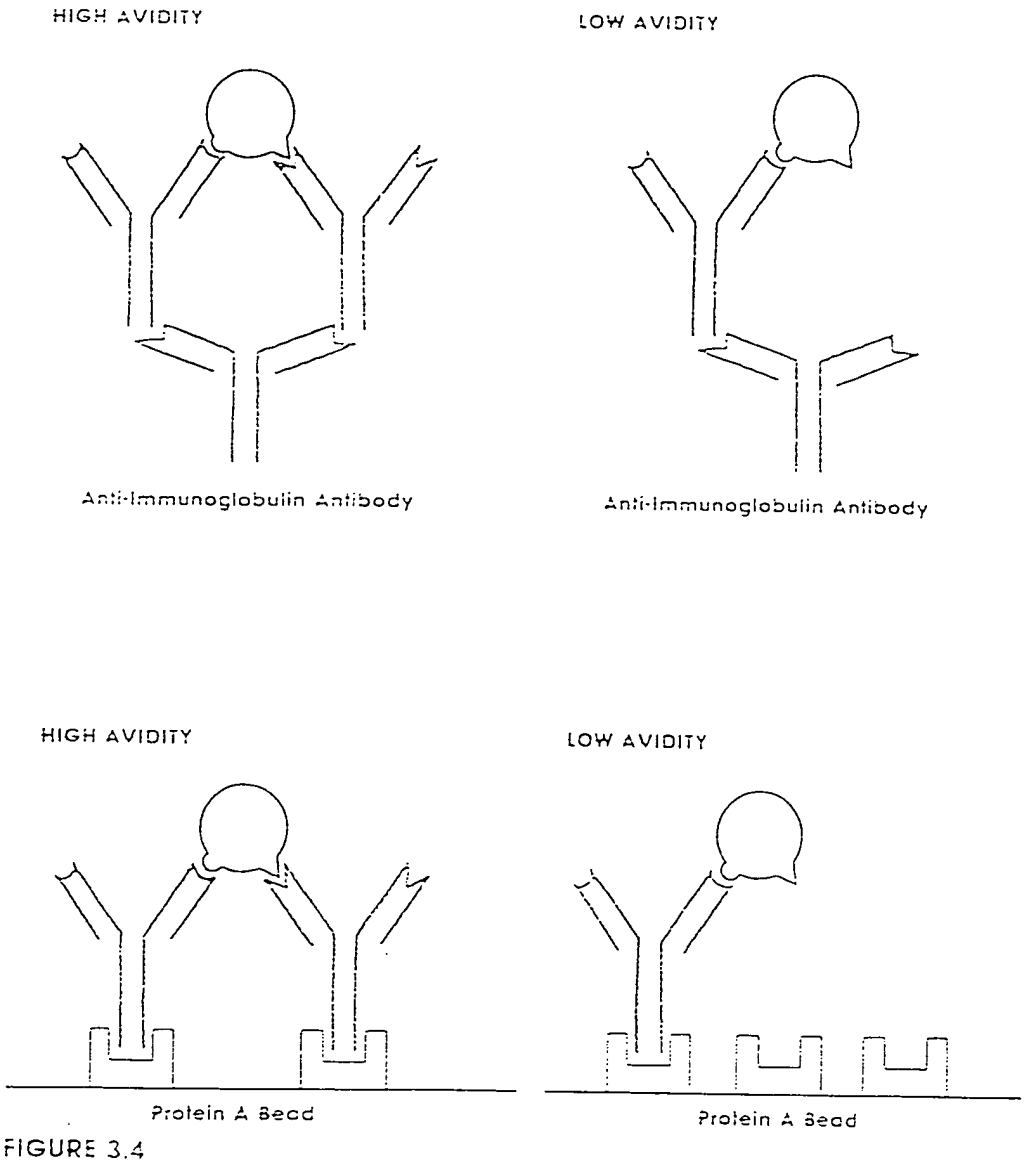

FIGURE 3.4
Polyclonal antibodies binding to multivalent antigens provide good targets for secondary reagents.

Antibodies that bind to multiple sites on an antigen provide an excellent target for secondary reagents

When an antigen is coated by many antibodies, the multimeric complex provides multiple binding sites in a flexible geometric arrangement that allows stable multimeric interactions with secondary reagents such as anti-immunoglobulin antibodies or protein A beads (Fig. 3.4).

This type of multimeric interaction can occur in most immunochemical techniques using secondary reagents.

Antigens immobilized on solid supports at high concentrations promote high-avidity, bivalent binding

When an antibody binds to an antigen on a solid phase, the interaction is biphasic, and two factors, in addition to the intrinsic affinity, control the strength of the interaction. These are the high local concentration of the antigen and the possibility of bivalent binding. The initial

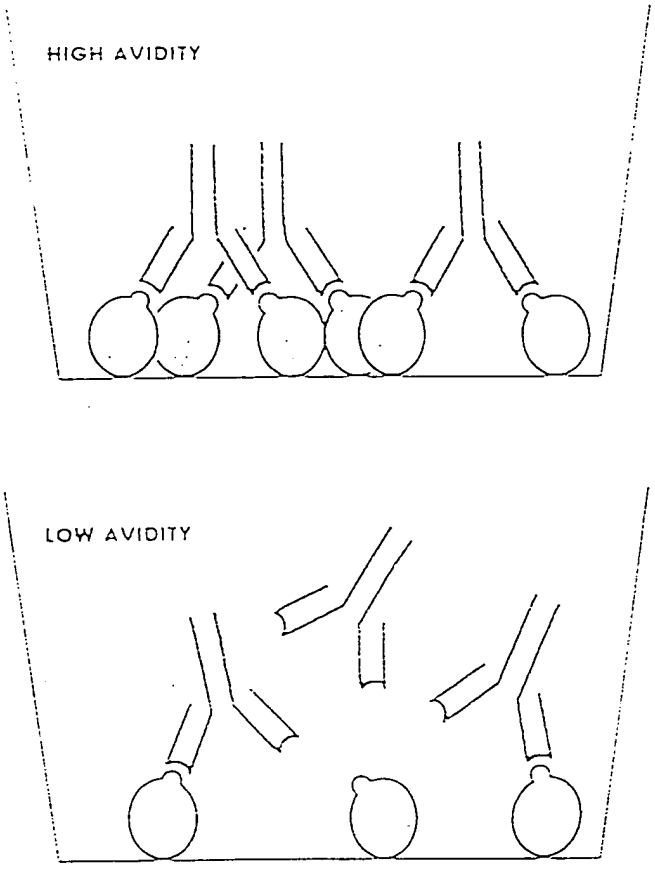

FIGURE 3.5
Bivalent binding to antigens immobilized on a solid phase increases the avidity.

binding of the antibody to the immobilized antigen is limited by diffusion, but after the first antibody–epitope interaction occurs, the formation of the second bond may be an intramolecular conversion if sterically possible (Fig. 3.5). In addition, the high local concentration of antigen increases the chance that any dissociated antibodies will rebind to neighboring antigens. In essence, diffusion occurs, but the high concentration of antigen acts as a trap to hold the antibody to the solid phase. These factors combine to yield a high avidity.

This type of multimeric interaction can occur in cell staining, immunoblotting, and many types of immunoassays.

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Immune Complexes As Antigens

Antigens purified by immunoprecipitation often show enhanced immunogenicity. If a source of antibody is available for the purification of an antigen, the compound can be purified readily and efficiently and used to immunize further animals. Purified immune complexes can be injected directly or can be injected coupled to beads. The presence of antibodies and/or the beads will stimulate phagocytosis of the antigen and usually will not harm the response. The antibodies should be as close as possible to the species used for the immunizations.

This method can provide a useful step in antigen enrichment for a wide variety of applications. It is particularly valuable when antibodies have been raised to one form of an antigen and now a different sort of reagent is needed. For example, the original antibodies may have been raised against a denatured antigen and now anti-native antibodies are needed. If there are epitopes displayed on the native antigen, then the antibodies can be used to collect a fully native antigen for further immunizations. This method can also be useful when using a monoclonal antibody as an intermediate to the preparation of a good polyclonal antisera. Injecting antibody-coated antigens has also been used to mask a particularly immunodominant epitope on an antigen, and thereby develop a response against other epitopes.

PREPARING IMMUNE COMPLEXES FOR INJECTION

The amount of antigen needed to elicit a strong response using immune complexes will vary from one compound to another. Doses of as low as 50 ng of antigen have been used successfully when delivered this way.

1. First titrate the amount of antibody needed to remove the antigen from solution. Add increasing amounts of antibodies to identical samples of antigen. Antibody can be added as either crude serum, monoclonal antibody ascites fluid, monoclonal antibody tissue culture supernatant, or purified antibody. In general, the purer the antibody preparation, the less chance of raising spurious anti-immunoglobulin antibodies.

2. After a suitable incubation time, process the samples as for an immunoprecipitation reaction (p. 429). Collect the immune complexes on protein A beads (p. 468).

3. Next titrate the amount of protein A beads needed to clear the antibody–antigen complexes from circulation. Prepare a large batch of antibody–antigen complexes using the ratios determined in step 1. Dispense in equal amounts and add increasing amount of protein A beads to each tube.

4. Incubate for 2 hr with rocking at 4°C. Process as usual for an immunoprecipitation.

5. After determining the appropriate amount of antibody and protein A beads, mix the antigen-containing solution with the appropriate amount of antibody. Incubate on ice for 2 hr.

6. Add the protein A beads. Incubate for 2 hr with rocking at 4°C.

7. Wash thoroughly. The beads can be collected by centrifugation or allowed to settle by gravity. Wash the beads four times by resuspension with the buffer in which the antigen was stored.

8. Run a small sample of the immune complexes on an SDS-polyacrylamide gel to check for purity. If the antibody–antigen immune complexes will be used directly to immunize animals, the beads and immune complexes can be injected as described on p. 103.

9. To elute the antibody–antigen complexes prior to injection, transfer the beads to a column.

10. Wash the beads with 10 column volumes of 100 mM Tris (pH 8.0).

11. Wash the beads with 10 column volumes of 10 mM Tris (pH 8.0).

12. Prepare a solution of 100 mM acetic acid (a 1 in 180 dilution of glacial acetic acid). Check the pH and adjust to approximately 2.5–3.0 by either adding more acetic acid or by diluting with water. Elute the column with the diluted acetic acid.

13. Identify the fractions that contain the immune complexes by using a spot test (p. 671 or 672). Neutralize the sample by adding NaOH or dialysis against PBS. If the samples are dilute and need to be concentrated, either neutralize or dialyze with a volatile buffer such as ammonium bicarbonate, and then lyophilyze.

14. Run a small sample of the immune complexes on an SDS-polyacrylamide gel to check for purity.

The immune complexes are now ready for injection (p. 92).

NOTES

i. If the antibodies used to purify the antigen do not bind well to protein A (p. 616), the buffers should be changed to the high salt variations described on p. 311 or the antibodies should be purified and covalently attached to the beads (p. 528).

ii. If the background is unacceptably high, any of the variations discussed in Chapter 11 can be used.

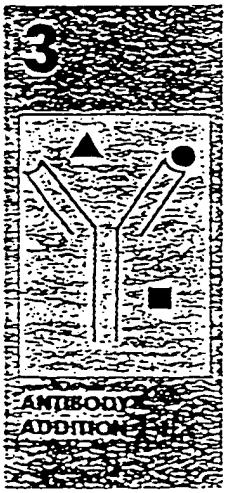
Forming the Immune Complexes

Forming an antibody–antigen complex is the simplest step in an immunoprecipitation. The variables to be considered are the amount of antibody to be added, the final volume, and whether or not an anti-immunoglobulin antibody will be used.

The amount of antibody to be added will depend on how much antigen will be precipitated. This can be determined by titrating the antibody versus a constant amount of antigen. With polyclonal antibodies, titrating the amount of antibody that is required to precipitate the antigen will often help to lower the nonspecific background. With monoclonal antibodies this will often not be necessary. For radiolabeled material, a general starting point will be 0.5–5 μl of a polyclonal sera, 10–100 μl of a hybridoma tissue culture supernatant, or 0.1–1 μl of an ascites fluid. At the midpoint of these three suggested ranges, the amount of heavy chain will be approximately 1 μg.

When using a polyclonal antibody, the volume of the reaction is seldom a concern. In fact, the avidity is normally high enough to allow efficient binding and eventually removal of the antigen from solution even with dilute samples. This feature means that the antibody–antigen interaction can be diluted with a solution containing nonspecific proteins. With monoclonal antibodies of high affinity, similar approaches are possible. However, with low-affinity monoclonal antibodies, using high concentrations of the antibody in low volumes will help drive the reaction.

Consult Tables 15.1 and 15.2 (pp. 617 and 618) to determine whether anti-immunoglobulin antibodies should be added. Polyclonal antibodies from human, rabbit, guinea pig, or pig never need to be supplemented; those from horse, cow, mouse, or hamster seldom; rat or sheep occasionally; whereas chicken or goat antibodies should always be supplemented. With monoclonal antibodies, rat subclasses should always be supplemented, and mouse subclasses other than IgG_{2a} should be supplemented for quantitative removal of the antibody. For quantitative precipitations, adding anti-immunoglobulin antibodies is recommended, but under some circumstances will increase the nonspecific background. When using anti-immunoglobulin antibodies, it is important to titrate the amount of the anti-immunoglobulin antibody that is needed to bind all of the primary antibody. This should be determined for each batch of antisera.



ADDING ANTIBODY TO LYSATES

1. Add serum (0.5–5 μl), hybridoma tissue culture supernatant (10–100 μl), or ascites fluid (0.1–1.0 μl) to a sample of lysate.

2. Incubate on ice for 1 hr. High-affinity reactions will be substantially complete considerably sooner than 1 hr; however, the 1-hr incubation time provides a reasonable compromise for most antibodies. Overnight reactions, although suggested in some protocols, are seldom any advantage, but will increase the background.

3. (Optional) If necessary add 0.5 μl of anti-immunoglobulin antibodies after 30 min. This can be conveniently added as 20 μl of a 1 in 40 dilution of whole sera.

Techniques for collecting the immune complexes are described on p. 466.

COMMENTS ◙ Controls

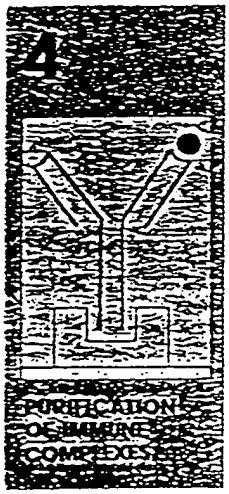
The correct controls for immunoprecipitation reactions should be antibody preparations that are as close to the specific antibody as possible. For example, polyclonal serum should be compared to other polyclonal serum from the same species. The best control will be a prebleed from the same animal used for immunization. For monoclonal antibodies, the control must be from the same source as the specific antibody, i.e., supernatant versus supernatant, ascites versus ascites, or pure antibody versus pure antibody. If possible, the control antibodies should be of the same class and subclass as the specific antibody. Tissue culture supernatants from the parental myeloma are never appropriate controls because they do not contain any antibodies. Suitable control hybridoma cell lines are available from ATCC.

EMBARASSING AND AVOIDABLE MISTAKES ARISE FROM THE IMPROPER CHOICE OF CONTROL ANTIBODIES (references available on request).

Purifying the Immune Complexes

All of the methods for purifying immune complexes rely on secondary reagents that bind to the antibody. The antibody is removed from the solution, and the antigen remains associated with the antibody throughout the purification. The earliest techniques used anti-immunoglobulin antibodies to form a large complex of antibody–anti-immunoglobulin antibodies. This multicomponent complex of molecules is known as a lattice. When the lattice is suitably large, it can be removed from solution by centrifugation. This procedure is still one of the best at yielding clean immunoprecipitations. However, the ability to form large enough complexes to be collected by centrifugation is critically dependent on the molar ratio of anti-immunoglobulin antibodies to primary antibodies. For quantitative removal of the immune complexes, the ratio must be determined empirically for each primary antibody that is used. To some degree, the size of the lattice will also depend on the amount and type of antigen. A large quantity of a multimeric antigen will change the potential size of any latticework compared with a rare, monomeric antigen. A monoclonal antibody will give a different set of problems to forming the lattice.

As a solution to these problems, Kessler (1975) suggested the use of protein A-bearing *S. aureus* Cowan I (SAC) as a solid phase to collect the antibody–antigen complexes. Protein A forms a portion of the cell wall of these bacteria. The cell wall proteins are fixed by treating with formaldehyde, the bacteria killed by heat treatment, and the resulting particles form an excellent solid-phase matrix to bind to the antibodies. The protein A binds to the Fc domain of the antibody, thus its attachment to the antibody does not affect the interaction with the antigen. The use of protein A and other cell wall proteins that bind antibodies is discussed in detail on p. 615. An alternative solid phase that can be used to lower some of the background problems encountered with SAC is a bead with purified protein A covalently attached. Techniques for both of these reagents are described below. Protein A beads give a cleaner background, but they are more expensive than SAC.



COLLECTING IMMUNE COMPLEXES ON FIXED S. AUREUS*

1. To the antibody–antigen reaction, add 50 μl of 10% fixed *S. aureus* Cowan I (SAC, wash the SAC twice in lysis buffer to remove any free protein A molecules from the solution and to transfer the SAC to the proper buffer). SAC is available commercially or can be prepared as described on p. 620.

2. Incubate on ice for 30 min.

3. Spin at 10,000*g* for 1 min at 4°C.

4. Remove the supernatant by aspiration and add 0.5 ml of lysis buffer.

5. Resuspend by vortexing. The SAC pellet will be difficult to resuspend, but placing two tubes in the vortex so they violently strike one another during vortexing will facilitate the resuspension.

6. Wash three times in lysis buffer. Remove the last wash as completely as possible. As some buffer will adhere to the sides of tubes, a quick spin to remove the last few microliters may lower backgrounds for critical applications.

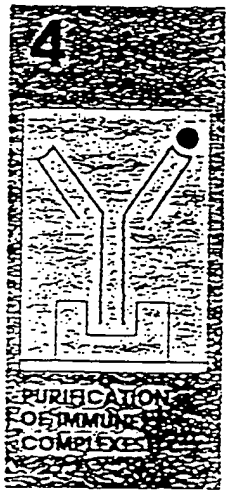
7. Use the immune complexes for the appropriate assay.

   For SDS-polyacrylamide gel electrophoresis, add 50 μl of Laemmli sample buffer (2% SDS, 10% glycerol, 100 mM DTT, 60 mM Tris [pH 6.8], and 0.001% bromphenol blue). Heat to 85°C for 10 min. Spin, remove supernatant, and load supernatant onto gel (p. 636). Samples can be stored at −20°C.

NOTE

i. If the SAC pellet is difficult to resuspend, several methods can help resuspension. Adding only 50 μl of lysis buffer prior to vortexing will help speed the resuspension. Vortex and then add the remainder of the 0.5 ml of lysis buffer, vortex briefly, and proceed. Freezing the pellet in a dry ice–alcohol bath prior to adding the wash buffer will also allow the cells to be resuspended quickly.

*Adapted from Kessler (1975, 1981).



COLLECTING THE IMMUNE COMPLEXES ON PROTEIN A BEADS

1. To the antibody–antigen reaction, add 100 μl of protein A beads (10% vol/vol in lysis buffer). Incubate 1 hr at 4°C with rocking.

2. Collect the beads by centrifugation at 10,000*g* for 15 sec at 4°C. Wash the immune complexes three times with lysis buffer. The lysate and wash buffers are easily removed by aspiration with a 23-gauge needle bent like this:

3. Remove the final wash as completely as possible. The needle should be inserted directly into the beads to remove the remaining wash buffer. Any beads that adhere to the needle can be removed by gently flicking it on the lip of the tube.

4. Use the immune complexes for the appropriate assay.

   For SDS-polyacrylamide gel electrophoresis, add 50 μl of Laemmli sample buffer (2% SDS, 10% glycerol, 100 mM DTT, 60 mM Tris [pH 6.8], and 0.001% bromphenol blue). Heat to 85°C for 10 min. Spin, remove supernatant, and load supernatant onto gel (p. 636) Samples can be stored at −20°C.